LI. THE ZINC CONTENT OF EPIDERMAL STRUCTURES IN BERIBERI

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The author [1938] has shown by calculation from the known Zn contents of a large variety of foodstuffs that the average amount of Zn ingested per day in a balanced diet is about 12 mg. and that this figure agrees with the average amount of Zn found by several workers to be eliminated daily in the faeces and urine of normal individuals. The author has also drawn attention to the fact that each of the three foods, sea fish, cooked polished rice and cabbage, which form the bulk of the diet of the poorest class of Chinese in South China, contains less than 6 mg. Zn per kg. of food as eaten. Since the amount of food consumed in a day by a person existing on such a restricted diet would not appreciably exceed 1 kg., it may be assumed that this represents the approximate daily Zn intake. Small additions of other foodstuffs richer in Zn will not materially augment this figure.

At this point it was realized that apart from containing little Zn, the three foodstuffs mentioned were also poor in aneurin. An examination of the available published data revealed strong evidence of a positive correlation between the Zn and aneurin contents of foodstuffs as shown in Table I and Fig. 1. For

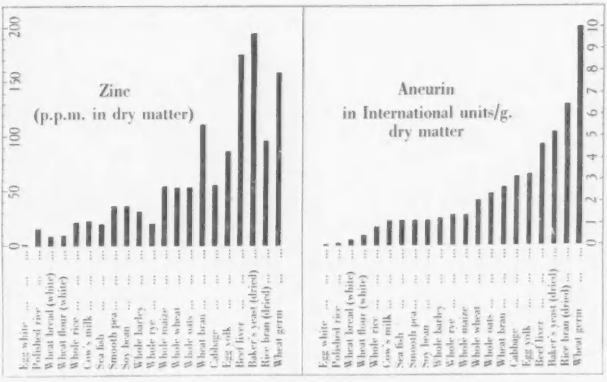

Fig. 1. Zinc and aneurin contents of foodstuffs.

this reason a diet supplying inadequate aneurin should also supply subnormal amounts of Zn and it would be reasonable to suppose therefore that sufferers from beriberi would show a reduction in the Zn content of their tissues.

Table I

Foodstuffs	Moisture† content %	Zn content* Fresh matter Range p.p.m.	Mean p.p.m.	No. of observations	Dry matter p.p.m.
Egg white	87·5	Nil–trace	Nil–trace	(4)	Nil–trace
Polished rice	15	9·7 – 14·5	13	(7)	15
Wheat bread (white)	33	4·1 – 8·0	5	(3)	8
Wheat flour (white)	20	6·5 – 8·0	7	(3)	9
Whole rice	15	14·65– 22·4	18	(3)	22
Cow's milk	86·5	2·07– 5·6	3·1	>(30)	23
Sea fish	80	0·3 – 8·0	4	(6)	20
Smooth pea	10	28·0 – 40·4	33	(4)	36
Soy bean	8	27·3, 38·0	33	(2)	36
Whole barley	15	—	27	(1)	31
Whole rye	15	—	17	(1)	20
Whole maize	54	—	25	(1)	55
Whole wheat	15	22·1 – 84·8	45	(5)	53
Whole oats	15	31·71– 77·5	45	(6)	53
Wheat bran	10	74·0 –139·2	100	(7)	111
Cabbage	94	2·6 – 3·7	3	(3)	55
Egg yolk	53	23·0 – 56·7	40	(8)	86
Beef liver	69*	34·5 – 84·0	54	(7)	175
Baker's yeast (dried)	—	36·4 –305	194	(4)	194
Rice bran (dried)	—	67·4, 70	69	(2)	69
Wheat germ	10	140, 145	142	(2)	158

Foodstuffs	Moisture† content %	Aneurin content‡ Fresh matter Range I.U./g.	Mean I.U./g.	No. of observations	Dry matter I.U./g.
Egg white	87·5	Nil–trace	Nil–trace	(2)	—
Polished rice	15	0·0 – 0·30	0·15	(4)	0·18
Wheat bread (white)	33	0·07 – 0·30	0·18	(18)	0·27
Wheat flour (white)	20	0·0 – 1·2	0·38	(11)	0·47
Whole rice	15	0·2 – 1·2	0·68	(21)	0·80
Cow's milk	86·5	0·06 – 0·25	0·15	(16)	1·1
Sea fish	80	0·0 – 1·0	0·22	(17)	1·1
Smooth pea	10	0·4 – 1·3	1·0	(4)	1·1
Soy bean	8	0·45 – 1·5	1·0	(8)	1·1
Whole barley	15	1·0 – 1·2	1·1	(4)	1·2
Whole rye	15	1·0 – 1·36	1·1	(3)	1·3
Whole maize	54	0·05 – 1·0	0·59	(5)	1·3
Whole wheat	15	0·75 – 3·40	1·7	(26)	2·0
Whole oats	15	1·1 – 3·25	1·9	(3)	2·3
Wheat bran	10	1·3 – 3·6	2·3	(6)	2·6
Cabbage	94	0·076– 0·25	0·19	(7)	3·1
Egg yolk	53	1·4 – 1·6	1·5	(2)	3·2
Beef liver	69	1·2 – 1·6	1·4	(4)	4·6
Baker's yeast (dried)	—	2·4 –10·0	5·1	(14)	5·1
Rice bran (dried)	—	4·2 – 7·6	5·8	(3)	6·4
Wheat germ	10	6·5 –22·0	90	(20)	10·0

* Derived from various sources quoted by Eggleton [1938].
† Derived from Read *et al.* [1937].
‡ Derived mainly from sources quoted by Fixsen & Roscoe [1938] but including also some values given by Pyke [1937].

Since epidermal structures are obtainable with equal ease from the living as from the dead and since they show little post-mortem change compared with internal viscera, it was decided to test the suggested correlation by investigating the Zn content of epidermal structures of patients with beriberi and normal individuals.

It is apparent that an abnormally low content of Zn in the epidermal structures might result also from low intake of food of normal Zn content. This starvation factor in the case of patients with beriberi cannot be ignored, but the correlation between the Zn and aneurin contents of foodstuffs shows that reduced epidermal Zn in beriberi is not wholly due to reduced food intake.

Methods

The samples were obtained partly from autopsies and partly from living persons, choice of material being governed by the need for keeping the comparison as unbiased and clear-cut as possible. It should be mentioned that in one case beriberi was complicated by amoebic dysentery with perforation which caused death. In another, a survival case, the beriberi had developed after cholera. The normal subjects were chosen from healthy living persons or from healthy accident cases or suicides showing no gross pathological changes. With three exceptions all samples were taken under the author's supervision.

The method of analysis used was essentially that described by Sylvester & Hughes [1936], which involves dry-ashing the clean, ether-extracted material, dissolving in HCl and extracting with dithizone at a specified *p*H. The Zn, after isolation from the dithizone, was estimated by the volumetric method of Lang [1929; 1933]. Several modifications making for increased accuracy and speed have been introduced and will be described elsewhere.

Results

The analytical results given in Table II show that the Zn content of the hair, nails and skin of patients with beriberi is about half that of those not suffering from the disease and that the ash contents of the nails and skin appear

Table II. *Zn and ash contents of epidermal structures in beriberi*

	Beriberi		Non-beriberi	
	Range	Mean	Range	Mean
Zinc content (p.p.m. of fat-free dry material)				
Head hair (pigmented)	110–229	173 (6)	84–444	225 (11)
Head hair (unpigmented)	145	—	198, 222	210 (2)
Beard hair	—	—	130, 167	149 (2)
Pubic hair	45–115	83 (4)	71–342	197 (8)
Finger nails	22–184	88 (13)	121–260	195 (13)
Toe nails	31–153	90 (6)	96–340	198 (11)
Skin (whole)	6– 21	13 (7)	12– 55	26 (8)
Epidermis	—	—	97	—
Ash content (% of fat-free dry material)				
Head hair (pigmented)	0·16–0·39	0·28 (7)	0·23–0·73	0·45 (10)
Head hair (unpigmented)	0·095 0·091	—	0·53, 0·72	0·63 (2)
Beard hair	—	—	0·31, 1·03	0·67 (2)
Pubic hair	0·18–0·70	0·51 (6)	0·10–0·75	0·40 (7)
Finger nails	0·22–3·35	1·18 (14)	0·07–0·56	0·30 (10)
Toe nails	0·42–1·53	0·68 (7)	0·15–0·72	0·39 (8)
Skin (whole)	2·33–3·90	2·92 (7)	1·37–2·56	2·08 (5)
Epidermis	—	—	0·12	—

Figures in brackets indicate number of subjects examined.

to be increased in beriberi. Why the ash content should be increased is not clear unless it be the result of diminished keratin formation arising from diminished protein intake. The determination of the ash in the small quantities of material

used is not so accurate as the determination of the Zn, so that less emphasis can be placed on the figures obtained, but the figures for Zn are clear-cut and leave no room for doubt.

Discussion

The idea of an association between the mineral content of a foodstuff and its aneurin content is not new. Schaumann [1908] pointed out that diets producing peripheral neuritis were invariably poor in phosphorus. In the erroneous belief that nucleic acid was the active principle he suggested that the P content of antineuritic substances was a direct measure of potency. Haag & Palmer [1928] have also drawn attention to the fact that some symptoms of aneurin deficiency are similar to those of Ca and P deficiency. Kilbourne [1910] has also pointed out that the potassium of rice meal is of diagnostic value as an indicator of its antineuritic potency, in which connexion it is interesting to note that Shiuza [1926] has claimed to have produced a beriberi-like disease in rabbits by a diet deficient in K. According to Bliss [1930] foods rich in vitamin B_2, such as beef, liver, egg-yolk and yeast (also rich sources of aneurin), are all iron-containing foods. Bliss has even gone so far as to suggest that pellagra is an iron deficiency disease since he obtained encouraging results from injection of iron in severe cases.

It would therefore appear likely that some of the symptoms commonly associated with beriberi and usually ascribed to aneurin deficiency may arise from, or be intensified by, deficiencies in certain elements commonly found in foodstuffs in association with aneurin. It is considered that Zn may have some importance in this regard. Curiously enough Hove *et al.* [1937] had found that rats fed on diets containing minimal amounts of Zn developed pellagra-like lesions round the eyes, at the mouth corners and on the feet and lost neck and shoulder hair. These abnormalities did not appear when the animals received a small supplement of Zn.

SUMMARY

In beriberi the Zn contents of the toe nails, finger nails and skin are reduced to half their normal value and there is a strong indication that the ash content is increased. Data are produced to show that the amount of Zn in natural foodstuffs is positively correlated with that of aneurin. It is suggested that Zn deficiency may be a factor in the beriberi syndrome.

I wish to express my gratitude to Dr L. T. Ride, Professor of Physiology in the University of Hong Kong, who very kindly gave me facilities for working in his laboratories.

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LII. INHIBITION OF DEHYDROGENASES BY SNAKE VENOM

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It has been reported previously [Chain, 1937, 1] that a substance, present in a number of different snake venoms, inhibits in very small concentrations the glycolysis and fermentation of cell-free extracts. The inhibitory effect of this substance is neutralized by the same quantity of antivenin as will neutralize the lethal effects of the venom *in vivo* [Chain, 1937, 2]. The chemical behaviour and mode of action of the inhibitory substance suggested an enzymic action causing the destruction of some integral part of the glycolysing and fermenting complex [Chain & Goldsworthy, 1937].

The following communication deals with the effect of snake venom on oxidation-reduction enzymes. This study was undertaken in the hope of throwing some light on the mechanism of the inhibitory action of the venom on glycolysis and fermentation, since oxidation-reduction enzyme systems play a predominant part in these processes.

Experimental

As in the previous experiments, dried venom of the Australian black tiger snake (*Notechis scutatus*) was used. The effect of this venom on various dehydrogenases and oxidases was studied.

Preparation of enzyme solutions. The lactic, malic, β-hydroxybutyric and succinic dehydrogenases and the cytochrome oxidase were obtained from pig's heart muscle. The muscle was minced twice through a Latapie mincer, washed exhaustively with tap water, ground with sand and $M/15$ phosphate buffer in a mortar and the solution strained through muslin [Ogston & Green, 1935]. The solution was brought to *p*H 7 and centrifuged for a short time. For some experiments a dried heart muscle preparation was used, prepared by dehydrating the minced washed pig's heart muscle with ice-cold acetone and evaporating off the acetone in a desiccator over P_2O_5. The dried powder (1·3 g.) was ground with sand and $M/15$ phosphate buffer *p*H 7·4 (20 ml.) in a mortar, the solution strained through muslin and centrifuged for a short time. The glycerophosphate dehydrogenase was prepared from rabbit's muscle [Green, 1936]. For xanthine oxidase untreated milk was used. The uric acid oxidase was prepared from sheep liver which was minced through the Latapie mincer, dried in a shallow dish *in vacuo* over P_2O_5 and ground in a mortar to a fine powder. The powder (1 g.) was extracted with borate buffer (10 ml.) *p*H 8·7 for 1 hr. at 37° and the suspension centrifuged. Amino-acid dehydrogenase was prepared from pig's kidney [Krebs, 1935].

Manometric experiments. The effect of the venom on the oxidative enzymes was tested in all cases by measuring O_2 uptakes in Warburg vessels at 37° in

O_2. In addition to 0·3 ml. 40% KOH for CO_2 absorption in the middle chamber, the vessels contained the following solution:

For lactic acid dehydrogenase:

Side bulb: 0·5 ml. 10% Na lactate.

Main space: 0·5 ml. 0·1% cozymase.[1]
0·3 ml. 5% semicarbazide hydrochloride, neutralized.
0·2 ml. 1% methylene blue.
1–2 ml. enzyme, according to activity.

For malic and β-hydroxybutyric acid dehydrogenases:

Side bulb: 0·5 ml. substrate (15·6% Na malate or 6·3% Na β-hydroxybutyrate)

Main space: 0·5 ml. 0·1% cozymase.
0·3 ml. *M*/10 Na pyrophosphate.[2]
0·3 ml. *M*/2 hydrazine sulphate, neutralized.
0·3 ml. 1% methylene blue.
1–2 ml. enzyme, according to activity.

For succinic and α-glycerophosphate dehydrogenases and cytochrome oxidase:

Side bulb: 0·5 ml. substrate (10% Na succinate, 4% ammonium α-glycerophosphate, 1% phenylenediamine hydrochloride, neutralized).

Main space: 0·5 ml. 0·1% cozymase.
0·2 ml. 1% methylene blue.
0·5–2 ml. enzyme, according to activity.

For xanthine oxidase:

Side bulb: 0·3 ml. 0·1% xanthine.

Main space: 1·7 ml. milk.
0·5 ml. *M*/5 phosphate buffer *p*H 7·4.

For amino-acid dehydrogenase:

Side bulb: 0·1 ml. 10% alanine.

Main space: 1 ml. water.
1 ml. enzyme.

For uric acid oxidase:

Side bulb: 0·3 ml. of a 1% suspension of uric acid in borate buffer *p*H 8·7.

Main space: 1 ml. water.
1 ml. enzyme.

Four Warburg vessels were used for each experiment—two control vessels containing the above solutions and two vessels containing snake venom in addition to these solutions. The amount of venom used was usually 1 mg. dissolved in 0·2 ml. water and it was placed together with the other solutions in the main space of the vessels.

[1] I am greatly indebted to Prof. R. A. Peters, Dr S. Ochoa and Dr L. A. Stocken, Department of Biochemistry, Oxford, for gifts of a cozymase preparation of approximately 90% purity.

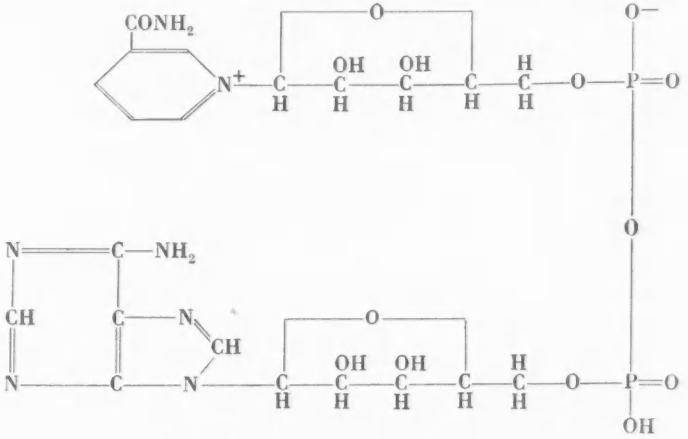
[2] In confirmation of the statement of Green *et al.* [1937] Na pyrophosphate was found to extend considerably the linear period of O_2 uptake with malic and β-hydroxybutyric dehydrogenases.

The results of the tests are summarized in Table I, the figures representing typical values of at least three sets of experiments.

Table I. *Effect of snake venom on oxidative enzymes*

Enzyme	O_2 uptake in 30 min. (μl.) Without venom	With venom	% inhibition
Lactic dehydrogenase	280	0	100
Malic dehydrogenase	180	0	100
β-Hydroxybutyric dehydrogenase	210	0	100
Amino-acid dehydrogenase	142	0	100
Succinic dehydrogenase	600	300	50
α-Glycerophosphate dehydrogenase	85	61	28
Cytochrome oxidase	417	276	34
Xanthine oxidase	167	175	0
Uric acid oxidase	296	308	0

It appears from Table I that lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases are completely inhibited by the venom; the other enzymes are not or only partially inhibited. The four inhibited dehydrogenases have one characteristic in common which differentiates them from the non-inhibited enzymes, namely the need for a coenzyme for their activation. The conclusion therefore naturally suggested itself that the inhibitory effect of the venom might be due to some action on the coenzymes. The need of a coenzyme for the lactic, malic and β-hydroxybutyric dehydrogenases has been known since the systematic study of their properties; it was found to be identical with coenzyme I, which according to Euler & Schlenk [1937] has the structural formula:



The existence of a coenzyme for amino-acid dehydrogenase was discovered only recently by Warburg & Christian [1937] who isolated it in form of its Ba salt and found it to be a flavin-adenine-nucleotide [1938, 1, 2].

By the following experiment it could be shown that coenzyme I is inactivated by black tiger snake venom. Coenzyme I (6 mg.) was dissolved in phosphate buffer *p*H 7·2 (2 ml.) and venom (2 mg.) in water (0·2 ml.) was added. The mixture was incubated for 1½ hr. at 37°. It was then brought to *p*H 1·5 with a

few drops of *N* HCl and incubated with cryst. pepsin (1 mg.) for 1 hr. at 37° in order to destroy the venom. The solution was then brought back to *p*H 7·2 and made up to 3 ml. A solution of coenzyme I (3 mg.) in phosphate buffer (2 ml.), serving as control, was incubated without the addition of venom for 1½ hr. at 37°, then adjusted to *p*H 1·5, incubated with cryst. pepsin (2 mg.) for 1 hr., brought back to *p*H 7·2 and made up to 3 ml. The coenzyme I activities of these solutions were tested with the malic acid dehydrogenase system. The arrangement of the test and its results are shown in Table II.

Table II. *Inactivation of coenzyme I by black tiger snake venom*

	Content of vessels in ml.		
	Vessel 1	Vessel 2	Vessel 3
Na malate 15·6%	0·5	0·5	0·5
Coenzyme I, 0·1% incubated as described above	0·2	0·2	—
M/10 $Na_4P_2O_7$	0·3	0·3	0·3
M/2 Hydrazine sulphate (neutralized)	0·3	0·3	0·3
Methylene blue, 1%	0·2	0·2	0·2
Coenzyme I, incubated with venom as described above	—	1·5	1·5
Water	1·5	—	—
Enzyme	1·0	1·0	1·0
O_2 uptake in 20 min. (μl.)	215	210	7

The O_2 uptake in vessel 3 is very small although it contained 1·5 ml. of the coenzyme I solution which, before incubation with the venom, contained 3·0 mg. Since 0·2 mg. of this cozymase preparation after the treatment described above was sufficient to activate the malic dehydrogenase, as shown by the O_2 uptake in vessel 1, at least 95% of it must have been inactivated by the venom during the incubation. The O_2 uptake in vessel 2 which is not essentially different from that of vessel 1 shows the effective destruction of the inhibiting substance in the venom by boiling and pepsin treatment.

An indication of the mechanism of the inactivation of coenzyme I by black tiger snake venom is obtained from the fact that CO_2 is evolved when snake venom is allowed to act on coenzyme I in $NaHCO_3$ solution.

Coenzyme I (2·4 mg.) dissolved in 0·5% $NaHCO_3$ (2·2 ml.) was placed in the main space of a Warburg vessel and snake venom (1 mg.) in water (0·2 ml.) and 0·5% $NaHCO_3$ (0·1 ml.) in the side bulb. The solutions were saturated with a gas mixture of 95% N_2 and 5% CO_2 at 37° and after establishment of equilibrium the venom solution was added to the coenzyme I solution. CO_2 evolution started immediately and ceased after 45 min. The total amount of CO_2 developed was 141 μl. and corresponds to 1·7 H^+ equivalents per mol. cozymase. The most likely explanation for this is a hydrolytic action of the venom on some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. Since one of these groups is bound to be weak and therefore will not be dissociated completely at the *p*H of the solution used in the experiment only 1·7 equivalents of CO_2 are measured instead of two.

The cozymase-inactivating factor in the snake venom which is responsible for the inhibition of glycolysis, fermentation and the four dehydrogenases needing coenzymes is thus shown to belong to the group of phosphoric acid-splitting enzymes, or more specifically, to the class of nucleotidases. It has been known for some time that certain Japanese snake venoms contain a phosphomonoesterase and a phosphodiesterase [Uzawa, 1932; Takahashi, 1932] and recently Gulland & Jackson [1938, 1] in an extensive study have found these enzymes in various proportions in a considerable number of different snake

venoms. The same authors report on the occurrence of an enzyme in several snake venoms which hydrolyses adenosine-5-phosphate and inosine-5-phosphate [1938, 2], and of a nuclease which hydrolyses yeast nucleic acid [1938, 3]. They suggested that some of the toxic effects of the venom might be due to the action of the 5-nucleotidase.

At present it cannot yet be decided by which of the several theoretically possible ways the hydrolysis proceeds. It was found that only a part (approx. 25 %) of the total P is set free in the form of inorganic P during the hydrolysis, and since the cozymase preparation was not 100 % pure it is possible that this inorganic P originates partly from some impurity present in the preparation and not from the cozymase. Obviously the amount of inorganic P liberated is too small to account for the 1·7 H^+ equivalents formed in the hydrolysis. Most probably the first step of the enzymic action is the opening of the pyrophosphate linkage in the coenzyme I molecule with the formation of two mono-ortho-phosphoric esters which then are partly broken down further with the liberation of inorganic P. It is hoped to get more information on this question from a study of the specificity of the phosphatases in the black tiger snake venom.

Summary

1. The effect of black tiger snake venom on the following oxidative enzyme systems has been investigated: lactic, malic, β-hydroxybutyric, amino-acid, α-glycerophosphoric and succinic dehydrogenases; cytochrome, xanthine and uric acid oxidases.

2. The four dehydrogenases which need a coenzyme for their activation, namely the lactic, malic, β-hydroxybutyric and amino-acid dehydrogenases, are completely inhibited by small amounts of black tiger snake venom. The others are not or only partially inhibited.

3. Coenzyme I is shown to be inactivated by black tiger snake venom. The inactivation is shown to be due to enzymic hydrolysis of some of the phosphoric acid linkages in the cozymase molecule with the formation of two new acid groups. The cozymase-inactivating principle in the black tiger snake venom can therefore be classified as a phosphatase or, more specifically, as a nucleotidase.

4. The inactivation of the coenzymes by black tiger snake venom through the action of a nucleotidase is the cause of the inhibitory effect of the venom on glycolysis, on fermentation and on the four dehydrogenases mentioned above.

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LIII. INVESTIGATIONS ON THE ROOT NODULE BACTERIA OF LEGUMINOUS PLANTS

XXII. THE EXCRETION PRODUCTS OF ROOT NODULES. THE MECHANISM OF N-FIXATION

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The excretion of N-compounds from leguminous root nodules has been the subject of many years' research in this laboratory. Apart from its great significance in nature and agriculture the excretion has also rendered possible a closer examination of the chemical processes of biological N-fixation which have been completely obscure. As established in several publications[1] from this laboratory the excretion in certain cases assumes such proportions that over 80 % of the total fixed N is excreted from the nodules to the medium by young legumes. This fact and the special composition of the excreted products show that the excreted N-compounds are products of N-fixation and not secondary products of protein decomposition. A thorough chemical investigation of the excretion products was therefore necessary for the elucidation of the mechanism of N-fixation.

The results of our studies hitherto on the chemical composition of excretion products have been described in brief preliminary communications and summarizing lectures. A detailed report of the methods of analysis and of the course of the research has, however, not been presented. In this paper we give such a report.

The biological aspects of the excretion of N-compounds will not be discussed in detail here; we refer in this respect to our earlier contributions.[2] From the general course of the work, however, the following may be mentioned. Peas were grown in a sterile culture system developed by us, using generally N-free quartz sand, and later also cellulose, as a medium. The composition of the nutrient solution was as follows: $Ca_3(PO_4)_2$ 0·25 g., $CaSO_4$, $2H_2O$ 0·25 g., $MgSO_4$, $7H_2O$ 0·39 g., KCl 0·25 g., $FeCl_3$ (5 % solution) 3 drops in 1000 ml. of distilled water. Peas were inoculated with an effective strain of legume bacteria isolated from the root nodules of pea (strain HX in this laboratory). The growth of the peas depended, in the absence of nitrogenous fertilizers, entirely on the N-fixation in the root nodules caused by the bacteria. In the sterile system the N-compounds excreted from the root nodules to the medium could decompose only through the action of the legume bacteria, and these decompose the excreted N-compounds only very slowly in a definite manner (cf. below). The

[1] The summary of the results obtained in this laboratory up to the end of 1937 in the field of N-fixation as well as the especially great bearing of legumes on agriculture and human nutrition have been described in A. I. Virtanen's lectures delivered in the University of London and now published in book form under the title, *Cattle Fodder and Human Nutrition with special reference to Biological Nitrogen Fixation*, Cambridge, 1938. References are also included in the book.

[2] Especially we refer to our articles in the *J. agric. Sci.* **27**, 332 and 584 (1937).

excretion products could therefore be isolated from the medium at the end of the experiment period unchanged, or changed in a definite manner.

In ordinary pot cultures, even when N-free medium is used for inoculated peas (or other legumes), the excretion products cannot be isolated as such, because various and numerous micro-organisms present in the medium decompose the excretion products thoroughly, forming ammonia and nitrate. The use of a sterile culture system is therefore a necessary condition for the chemical elucidation of excretion products.

The plant experiments have been carried out with Dr Synnöve v. Hausen.

Experimental

Extraction and characterization of the excretion products

The experiment in 1932 [Virtanen *et al.* 1933] showed that when inoculated peas were grown in quartz sand in sterile culture system, without N-nutrition, and the N-compounds excreted into the medium were isolated by removing the roots carefully from the same, and extracting the sand with boiling water, the extract obtained contained the following N-fractions (Table I).

Table I

	N, mg.	N, % of total N
Total N in the extract	61·50	100·00
Amino-N	47·60	77·40
Ammonia-N	0·00	0·00
Amide-N	2·03	3·30
Volatile bases-N	1·68	2·73
Melanin-N	1·26	2·05

This preliminary experiment already showed that the major part of the nitrogen was amino-N. Since hydrolysis with HCl did not increase the amount of amino-N, the solution contained only free amino-acids and no peptides. Among the individual amino-acids an attempt was made to determine aspartic acid but with negative result. As was noticed later, this failure was due to the fact that the determination was made in the solution after ammonia determination according to Van Slyke, when aspartic acid is precipitated with alcohol and removed from the solution. The presence of volatile bases and melanin-N in the extract led to the assumption that the extraction with boiling water had probably caused decomposition in the primary excretion products.

We therefore made experiments with another excretion method. In a continuously acting apparatus sand was placed in a tinned metal dish, capacity 60 l. A glass tube near the bottom of the dish was led into a boiling flask, in which the water, acidified with H_2SO_4 to *p*H 2–3, was kept boiling. Steam was passed through a condenser, from which the water dropped on to the sand in the metal dish. In the flask the extracted compounds remained in the boiling solution for several days as the extraction in this apparatus was slow. Even this method therefore offered great possibilities for the decomposition of N-compounds. This extraction method was employed in the following experiment.

In the spring of 1935 peas were grown in our greenhouse in 25 Woulff's bottles (3 l.) each containing 4 kg. quartz sand. Two inoculated peas were grown in each bottle. The cultures were harvested in the middle of the flowering period, and the sand was washed from the bottles using as little water as possible. The root particles were carefully removed from the sand. The sand from all bottles (100 kg.) was extracted together for about 100 hr. in the manner

described. The extract was concentrated *in vacuo* to 500 ml. and filtered. The small precipitate contained 10 mg. N, and the clear filtrate, extract II, 577·5 mg. N. N was determined in both cases by the Kjeldahl method.

The filtrate contained the following amounts of amino-N [Van Slyke]:

After 5 min. reaction time:

In 10 ml. (1) 9·55 mg.
In 10 ml. (2) 9·49 mg. } In 500 ml. 476·0 mg. amino-N.

After 30 min. reaction time:

In 10 ml. (1) 10·05 mg.
In 10 ml. (2) 10·10 mg. } In 500 ml. 504 mg. amino-N.

The increase of amino-N during 30 min. reaction time indicates that the filtrate apparently contained also some other N besides α-amino-N.

Volatile bases were determined in 25 ml. of the solution according to Van Slyke's distillation method. The distillate used 1·1 ml. of 0·1 *N* H_2SO_4 corresponding to 1·54 mg. NH_3-N.

Ammonia-N was determined by the same distillation method also in 25 ml. Ammonia was determined in the distillate by Nessler's reagent. 0·3 mg. NH_3-N was found. Accordingly:

500 ml. contained 6·0 mg. NH_3-N,
500 ml. contained 24·8 mg. N of other volatile bases.

Amide-N was determined in the solution after volatile bases had been distilled according to Van Slyke. Conc. HCl was added to the solution up to 20 %. The mixture was heated on the water bath for 14 hr. after which the major part of the HCl was evaporated *in vacuo*. The residue was nearly neutralized with 20 % NaOH, made alkaline with MgO and NH_3 was distilled over. The distillate used 0·8 ml. of 0·1 *N* H_2SO_4 corresponding to 1·12 mg. NH_3-N. 500 ml. thus contained 11·2 mg. "amide-N".

Table II illustrates the quantities of different N-fractions in extract II.

Table II

	N, mg.	N, % total N
Total N	577·5	100·0
Amino-N	504·0	87·3
NH_3-N	6·0	1·0
Volatile bases-N	24·8	4·3
"Amido-N"	11·2	2·0

In this extract the amount of amino-N was considerably higher than in the extract of the preliminary experiment. The extraction method seemed thus to have a marked effect on the composition of the N-compounds. Since in extract II also the N-compounds remained for a long time in boiling solution at *p*H 2–3, decompositions were still possible.

In extract II the following qualitative tests and determinations were made:

1. Ninhydrin reaction was positive.
2. Folin's amino-acid reaction with 1:2-naphthoquinone-4-sodium sulphonate was positive.
3. Klein's arginine reaction was negative.
4. Winkler's tryptophan reaction was negative.
5. The determination of organic sulphur was negative; thus sulphur-containing amino-acids were not present.
6. Pauly's diazo reaction for histidine and tyrosine was negative.

7. Millon's reaction was negative.

8. The enzyme aspartase (dry preparation of propionic acid bacteria) formed ammonia abundantly in the extract. According to our experiments the said dry preparation does not split off ammonia in the presence of toluene from any amino-acids except from *l*-aspartic acid. Therefore it was evident that *a large quantity of aspartic acid was present* in the extract. Quantitative determination was not made.

9. 100 ml. of the extract (=115·5 mg. N) neutralized to *p*H 7 were extracted for 100 hr. with *n*-butyl alcohol in Dakin's vacuum extraction apparatus. Only 0·5 mg. N was removed by the butyl alcohol. Thus it was evident that mono-amino-monocarboxylic acids and proline were not present in the extract.

10. A test was made to find out whether a part of the N in the extract was precipitable with phosphotungstic acid. 50 ml. of the extract (=57·8 mg. N) were neutralized with NaOH, whereupon 15 g. of phosphotungstic acid were added and the solution brought to the boiling point. The solution was kept for 48 hr. at 37° before the precipitate was separated. Another sample of 50 ml. was taken and 10 ml. of conc. HCl and 15 g. of phosphotungstic acid were added. The solution was kept in the ice-box for 48 hr. N determinations in the precipitates of the two experiments gave the following results:

(1) 24·0 mg. N or 42 % of total N,
(2) 28·6 mg. N or 49·7 % of total N.

Since a considerable part of the N was precipitated with phosphotungstic acid, it was evident, in view of the above observations, that the extract contained, in addition to aspartic acid, either diamino-acids or other non-α-amino-acids.

Extract II already contained nearly 90% of the total N in the form of amino-N. The presence of melanin-N and volatile bases indicated that some decomposition had probably taken place during the extraction in boiling solution. Therefore we employed in the following extraction a new method, in which all heating was prevented. This procedure, which we have since used regularly, is illustrated in Fig. 1.

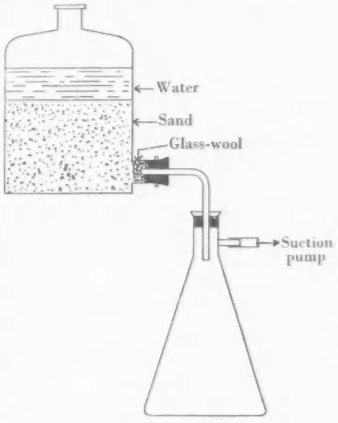

Fig. 1.

The sand from 10 Woulff's bottles, each containing 4 kg. quartz sand and 2 inoculated peas grown in summer 1935 without N-nutrition, was washed from the bottles with a small amount of water and the roots were removed simultaneously. The sand was extracted in portions of 8 kg. with cold water (about 15–20°) in the apparatus shown in Fig. 1. 3 l. of water acidified with H_2SO_4 to about *p*H 4 and containing some toluene were mixed well with the sand, whereupon the water was sucked into a filter flask. The extraction was repeated 10 times with the same water. Then a new washing was carried out in the same manner with another 3 l. of water. Each 8 kg. portion of sand was thus finally washed with 6 l. of acid water containing toluene, and the total amount of 40 kg. of sand with 30 l. of water. In this manner 80 % of the excreted N was recovered from the sand (cf. below).

The solution was evaporated to 500 ml. *in vacuo* on the water bath at 40°. The small precipitate formed during the evaporation was filtered off, and the filtrate was poured into a 500 ml. measuring glass and made up to this volume with water (extract III). 500 ml. of the extract contained (Table III):

Table III

	N, mg.	N, % of total N
Total N	259·0	100·0
Amino-N (30 min. shaking time)	256·0	98·8
NH_3-N	Traces	—
Volatile bases-N	Traces	—

The extraction at lower temperature showed that the N excreted from the root nodules was almost entirely amino-N and that the other N-compounds found in the previous extracts were evidently ascribable to the decomposition processes occurring during the extraction.

In this extract the same qualitative determinations were first made as in that described above. The results were identical.

Isolation and characterization of individual excretion products

Isolation of aspartic acid. 5 ml. of 10 % milk of lime and 250 ml. of 96 % alcohol were added, with good stirring, to 20 ml. of extract (= 10·36 mg. N), and kept for 1 hr. (Foreman's precipitate). The mixture was filtered and the precipitate washed carefully with 90 % alcohol, whereupon N-determination was made. The precipitate contained 5·25 mg. N or 50·7 % of the total N. The extract thus contained a monoamino-dicarboxylic acid. Attempts to identify glutamic acid as hydrochloride and as Zn salt, gave negative results. On the other hand we succeeded in isolating aspartic acid as the Cu-salt in the following manner. Amino-acids were precipitated from 50 ml. of extract (= 25·9 mg. N) according to Neuberg. The extract was made alkaline with 10 % Na_2CO_3. Small amounts of 25 % Hg acetate and 10 % Na_2CO_3 were added alternately so that the solution was kept constantly alkaline; 6 vol. of alcohol were added, the solution was filtered and the precipitate suspended in water and decomposed with H_2S. The filtrate was evaporated *in vacuo* to a small volume (about 25 ml.) and boiled with freshly precipitated $Cu(OH)_2$. It was filtered hot and kept in the ice-box. After a while the Cu-salt began to precipitate. Filtration was carried out after some hours, the precipitate was carefully washed with cold water and dried in a desiccator over H_2SO_4. The Cu-salt weighed 150 mg. Cu was determined according to Abderhalden & Schnitzler [1927] and N according to micro-Kjeldahl. (Found: Cu, 22·97, 23·30; N, 5·10, 5·03 %. $C_4H_5O_4NCu$, 4·5 H_2O requires Cu, 23·06; N, 5·08 %.)

The salt obtained was thus the *Cu salt of aspartic acid.* To confirm the fact, aspartic acid was also determined in the Cu salt by means of the enzyme aspartase.

190 mg. Cu salt were dissolved in 25 ml. of 0·1 *N* H_2SO_4 and Cu was precipitated with H_2S. H_2S was evaporated from the filtrate. 10 ml. of *M*/15 phosphate buffer (*p*H 7), 10 ml. of a suspension of *B. fluorescens liquefaciens* (from 375 mg. of dry bacterial preparation) and 10 ml. of toluene were added. The volume of the solution was made up to 150 ml.

The parallel solution consisted of 100 mg. of *l*-aspartic acid, 10 ml. of phosphate buffer, 10 ml. of bacterial suspension and 10 ml. of toluene, made up to 150 ml.

The control solution for the determination of the NH_3-content of the bacterial suspension was otherwise the same as the parallel solution, but did not contain aspartic acid.

All three experimental solutions were kept for 5 days at 37°, after which NH_3 was determined. According to our experience liberation of ammonia from aspartic acid by aspartase reaches practically its maximum during this time, and the reaction *l*-aspartic acid $\rightleftarrows$ fumaric acid + NH^3 attains its equilibrium.

The following amounts of ammonia were found in the different solutions:

	Bacterial suspension without aspartic acid	Bacterial suspension + 100 mg. *l*-aspartic acid (= 10·53 mg. N)	Bacterial suspension + filtrate corresponding to 190 mg. Cu salt (= 9·69 mg. N)
NH_3-N, mg.	4·76	10·50	9·80
NH_3-N, formed from the substrate	—	5·74	5·04
NH_3-N, % of the N in the substrate	—	54·50	52·00

The data show convincingly that the isolated Cu salt was Cu *l*-aspartate. It is known that aspartase does not split ammonia from *d*-aspartic acid.

Several methods were used for the quantitative determination of aspartic acid in the extract. The aspartase method gave the following result.

40 ml. of the extract (= 20·01 mg. N) + 10 ml. of a suspension of *B. fluorescens liquefaciens* (prepared from 375 mg. of dried bacteria) + 10 ml. of *M*/15 phosphate buffer (*p*H 7) + 10 ml. of toluene were made up with water to 100 ml. During 5 days at 37°, 5·6 mg. NH_3-N were formed (control, with bacterial suspension alone, subtracted). Since, under the same experimental conditions with the same bacterial material, *l*-aspartic acid forms 54 % NH_3-N of the total N, the amount formed in the extract, 5·6 mg. NH_3-N, corresponds to 10·4 mg. *l*-aspartic acid. Consequently, *52 % of the total N in the extract (= 20·01 mg. N) was* l-*aspartic acid-N*.

When amino-dicarboxylic acids were precipitated from the extract according to Foreman as the Ca salts, 50·7 % dicarboxylic acid-N was found. Since the aspartase method gave 52 % aspartic acid-N of the total N, it was evident that *the whole Foreman-fraction was* l-*aspartic acid*.

Aspartic acid was determined in still another way, viz. by oxidizing it first to malic acid and further to acetaldehyde. According to Fürth *et al.* [1932], the extract was treated with nitrous acid, followed by permanganate oxidation (the ordinary lactic acid determination). Acetaldehyde is only formed in this manner from α-alanine and aspartic acid. The acetaldehyde formed corresponded to 48·5 % of the total N in the extract. In the absence of α-alanine this N belongs to *l*-aspartic acid.

2 ml. of extract + 75 ml. of water + 0·5 ml. of conc. HCl were heated on the water bath. 15 ml. of 2·5 % $NaNO_2$ were added drop by drop from the burette during 20 min., after which 15 ml. of 7·5 % urea were added in the same manner to remove the excess of nitrite. The solution was made up to 250 ml. and the oxidation with permanganate was accomplished in 50 ml.

1·80 ml. of 0·008 *N* iodine solution were used, corresponding to 0·317 mg. acetaldehyde. From the whole extract (500 ml. = 259 mg. N) 396·5 mg. acetaldehyde were formed, corresponding to 126 mg. N or 48·5 % of the total N.

The different methods, Foreman's precipitation, liberation of ammonia by aspartase and the oxidation of aspartic acid after treatment with nitrous acid, thus gave the following amounts of aspartic acid.

Foreman's precipitation ...	50·7 %	aspartic acid-N of the total N
Aspartase method	52·0	,, ,,
Oxidation to malic acid and further to acetaldehyde ...	48·5	,, ,,

The results of the different methods are in good agreement. Thus approximately 50 % of the N in the whole extract is aspartic acid-N. As was shown by the aspartase method, the aspartic acid in question is the *l*-form. Glutamic acid was not found in the extract.

Characterization of the amino-acid precipitable with phosphotungstic acid

Nearly all of the rest of the N in the extract was precipitated with phosphotungstic acid. 50 ml. of the extract (=25·9 mg. N) were precipitated in acid solution with 15 g. of phosphotungstic acid. The solution was heated to boiling and then kept in the ice-box for 48 hr. 12·0 mg. N were precipitated or 47·1 % of the total N. Aspartic acid-N together with the N precipitated with phosphotungstic acid thus formed 97–99 % of the total N. On the basis of the qualitative experiments described above it was evident that the N precipitated with phosphotungstic acid belonged either to a diamino-acid or to a non-α-amino acid. In order to elucidate this point some additional determinations were made.

Formaldehyde titration. 8 ml. of the extract, freed from phosphoric acid and CO_2, used 2 ml. of 0·1 *N* NaOH corresponding to 2·8 mg. amino-N. The whole amount of amino-N was 4·10 mg., thus only 68·3 % of the amino-N had been fixed through formalin. In view of this fact a part of the amino-N must be in a position other than α with regard to carboxyl.

Carbon determination in the extract was made according to Osburn & Werkman [1932]. 25 ml. of extract (=12·95 mg. N) gave 156·0 mg. CO_2, whence the atomic C : N ratio was 3·8 : 1. In aspartic acid this ratio is 4 to 1, and consequently this ratio in the remaining amino-acid or acids in the extract must be smaller than 3·8 : 1, and in fact considerably smaller because the extract contains also some N-free organic compounds (cf. below).

Absorption spectrum. Amino-acids were precipitated according to Neuberg. The mercury precipitate was decomposed with H_2S, and the solution was evaporated *in vacuo* to a small volume. The final solution contained 10 mg. N in 100 ml. The absorption spectrum in the ultraviolet was photographed. No absorption maxima were noted, showing the absence of tryptophan, tyrosine and phenylalanine. A strong end absorption occurred rising abruptly at 230 mμ but this is characteristic of all aliphatic amino-acids.

Preparation of a picrate from the amino-acid fraction precipitable with phosphotungstic acid did not succeed. A dilute alcoholic solution of picric acid formed a precipitate, but it contained mostly potassium picrate, and no pure and characteristic amino-acid picrate could be isolated.

Taking into account all the facts hitherto observed, there was reason to assume that the amino-acid precipitable with phosphotungstic acid was a diamino-acid, probably lysine, provided that the amino-acid in question was a normal amino-acid present in proteins. Attention was therefore paid to the determination of lysine. This amino-acid is difficult to characterize and is therefore often determined indirectly. In order to develop a specific method for the determination of lysine we considered the possibility of splitting the carboxyl group from the lysine with the formation of cadaverine, which is easy to determine. As we have previously reported [1937], an almost quantitative production of CO_2 from lysine was accomplished by a strain of *B. coli* and the corresponding amount of cadaverine was obtained. Applying the same method to our extract we could not obtain cadaverine. From one of our extracts (extract IV), which contained 32·3 mg. N in 30 ml., we precipitated aspartic acid as the Ba salt according to Foreman. The precipitate contained 15·54 mg. N or 48·3 % of total N. Alcohol was evaporated from the filtrate and Ba was removed

as sulphate. The obtained filtrate was evaporated to 25 ml., 2 g. of moist *B. coli* (aseptically isolated) and 25 ml. of glycerol nutrient solution were added. No formation of cadaverine occurred within 3 weeks at 37°. From the parallel experiment, to which lysine was added, 90% of the theoretical amount of cadaverine was isolated. This proved that the N-fraction precipitating with phosphotungstic acid could not contain lysine. Putrescine, which is formed from ornithine by the same bacteria, was also excluded.

In the absence of all the known α-amino-acids, which might possibly have passed into the N-fraction precipitable with phosphotungstic acid, the unknown amino-acid must be one which is not found among the products of protein hydrolysis. Our attention was now directed to β-alanine. It appeared that the *legume bacteria split off quantitatively one carboxyl group from* l-*aspartic acid with the production of* β-*alanine* [Virtanen & Laine, 1937]. The presence of β-alanine among the excretion products of root nodules was thus quite natural. The following methods were used for the identification of β-alanine in our extracts.

The formation of ethyl acrylate. All the extracts investigated, and their N-fractions precipitable with phosphotungstic acid, gave, when treated with HCl and alcohol according to Abderhalden & Fodor [1913], a strong smell of ethyl acrylate. β-Alanine can be qualitatively determined in this manner.

The butyric acid compound of β-*alanine.* Przylecki & Kasprzyk [1937] have introduced a method for the characterization of basic amino-acids by means of their butyric acid compounds. 100 ml. of the extract (*p*H about 4) from sterile sand cultures of inoculated peas, containing 69·0 mg. N, were extracted in a percolator with ether for 3 days to remove the ether-soluble substances (cf. below). The aqueous solution, containing all the amino-N, was evaporated *in vacuo* almost to dryness, whereupon anhydrous Na_2CO_3 and anhydrous Na_2SO_4 were added. The dry mass was extracted in a Soxhlet apparatus with abs. methyl alcohol for 48 hr. 28·1 mg. N or 40·7% of the total N in the extract were obtained in the methyl alcohol, which was evaporated to dryness *in vacuo*. The residue was extracted with abs. butyric acid for 4 hr. and then filtered. The butyric acid solution contained 20 mg. N. Ether was added to the solution, causing the formation of a fluffy precipitate containing N. On washing with ether the precipitate became syrupy. It was dissolved again in butyric acid and precipitated once more with ether. The precipitate was washed with ether and dried in a desiccator. Its M.P. was about 180°. The butyric acid compound prepared from β-alanine melted at 204°. The mixture of both melted at 190°. On microscopic examination the butyric acid compound prepared from the extract was found to contain crystals typical of the butyric acid ester of β-alanine. A part of the preparation was, however, amorphous and the low M.P. was evidently due to this. Various salts in the extract originating from the nutrient solution of peas, were partly dissolved in methyl alcohol and butyric acid, and obviously prevented complete purification of the butyric acid compound.

The butyric acid compound gave, when treated with HCl and alcohol, a distinct smell of ethyl acrylate.

Oxidation of the extract with ninhydrin. α-Alanine is quantitatively oxidized with ninhydrin to acetaldehyde; aspartic acid forms at the most 10% of the theoretical amount; other normal amino-acids do not form acetaldehyde, whereas β-alanine forms 10–34% of the theoretical amount in different experiments [Virtanen & Laine 1938].

From an extract of quartz sand cultures of peas (extract V) aspartic acid was removed according to Foreman. The filtrate which contained 12·1 mg. N gave, on oxidation with ninhydrin, 6·46 mg. acetaldehyde or 17% of the theoretical

amount (1 N atom corresponds to 1 mol. acetaldehyde). This result therefore supports the conclusion that β-alanine forms at least the major part of the N-fraction precipitable with phosphotungstic acid.

Whether the whole fraction consists of β-alanine does not directly appear from our experiments. The absence of all known amino-acids implies, however, that β-alanine is the only amino-acid in the fraction. This fact is confirmed also by the ratio of aspartic acid to the fraction precipitable with phosphotungstic acid in the sand during different stages of growth of the pea (cf. below).

Nitrite- and oxime-N in the extract

As has been mentioned above, the extraction of quartz sand cultures of inoculated peas with acid water at room temperature produces extracts in which nearly all (90–99 %) the N is amino-N as determined according to Van Slyke using 30 min. reaction time. In the autumn of 1935 we detected the presence of NO_2-N in the extract by means of α-naphthylamine and sulphanilic acid according to Blom [1926]—later we used the more sensitive reagent atoxycocaine instead of sulphanilic acid according to Jendrassik & Falcsik-Szabó [1933]. Before extraction also some nitrite was generally found in the sand cultures. In every case, however, the NO_2-content of the concentrated extract was many times higher than before extraction of the sand. During extraction and especially during concentration of the extract some N-compounds must thus have formed NO_2-N. This is evident from the following experiment.

Sterile system. 2 Woulff's bottles; 4 kg. quartz sand; 2 inoculated peas in each bottle. N-free nutrient solution. After 40 days' growth the plants were harvested and roots carefully removed from sand.

4 peas		
Dry wt., g.	N, mg.	N, excreted in sand, mg.
2·040	60·6	90·9

In this experiment much more N was excreted into the sand than had been taken up by peas. Solution sample taken from the sand gave a very slight nitrite reaction.

The sand was washed five times, in the apparatus described above, with a total of 16 l. tap water. After every washing NO_2-N was determined in the washings, which were then evaporated *in vacuo* to 100 ml. The air passed through the solution during evaporation was washed with alkali to remove possible N-oxides. The washing waters contained the following amounts of NO_2-N:

1.	4 l.	washing water	0·16 mg. NO_2-N
2.	3	,,	0·15 ,,
3.	3	,,	0·05 ,,
4.	3	,,	0·01 ,,
5.	3	,,	0·00 ,,
		Total	0·37 mg. NO_2-N

100 ml. of concentrated extract contained 1·72 mg. NO_2-N; thus its amount had increased during concentration from 0·37 to 1·72 mg. Also during the washing of the sand a considerable increase of NO_2-N had already occurred, but the amount cannot be expressed quantitatively, since the determination of nitrite in sand was not quantitative.

Examining the course of formation of NO_2-N we noticed that Endres [1935] had recently found oxime-N in *Azotobacter* cultures. Employing this method (hydrolysis of the oxime at 100° for 6 hr. in 3 *N* H_2SO_4, followed by oxidation of the hydroxylamine formed with iodine to nitrite at room temperature) we found oxime-N in our extract also. In sand cultures it usually amounted to 1–2 % of

the total excreted N. NO_2-N was apparently formed from oxime-N, since in the concentrated extract oxime-N was decreased and NO_2-N increased. Later, when the chemical nature of the oxime was known, it was possible to prove experimentally the formation of NO_2-N in sterile cultures of peas from the oxime in question (cf. below).

Since the oxime is partly decomposed during slow extraction and particularly during long vacuum evaporations of large volumes of liquid, we started to use finely divided cellulose as a medium for the peas in order to isolate the oxime. 500 g. finely divided cellulose were placed, together with a sufficient amount of N-free nutrient solution, in a 1·5 l. suction flask. Two peas were grown in each flask. The sterile system was the same as with sand. All the excreted N-compounds were removed from the cellulose by 2–3 washings with a little water. In our experiments the nutrient solution was first pressed from the cellulose, after which the latter was washed three times with 1·5 l. of distilled water, altogether 4·5 l. The washings were added to the nutrient solution and evaporated as rapidly as possible *in vacuo* to 100 ml. Even then decomposition of oxime occurred to some extent as indicated by the following experiment.

Two parallel experiments with inoculated peas. Period of growth, 1 Mar.–23 April, 1936. Cellulose used as a medium.

	Peas		Medium			
No. of exp.	Dry wt., g.	N, mg.*	Excreted N, mg.	Oxime-N mg.	NO_2-N, mg.	Oxime-N + NO_2-N % of excreted N
1	1·370	36·5	7·3	0·06	0·10	2·2
2	1·291	34·4	6·7	0·12	Traces	1·8

* N in seeds is subtracted.

The total amount of oxime-N and nitrite-N in numerous cellulose cultures investigated was regularly 1–2 % of the excreted N. Only in one experiment did this amount rise to 10 %. In cultures of ripening peas, when N-fixation in the root nodules ceases, oxime-N is no longer found. Amino-N amounted, in cellulose cultures, to 95–98 % of the excreted N.

The isolation and characterization of the oxime was accomplished as follows.

Isolation and characterization of oxime

Isolation as Cu salt. From several cellulose cultures of peas the N excreted from the root nodules was extracted with water in the manner outlined above. 200 ml. of concentrated extract were made acid and extracted for 24 hr. in a percolator with ether, which removed 4 mg. oxime-N. The ether was evaporated of and the residue dissolved in a little water. A small amount of this solution was precipitated according to Foreman; all the oxime-N was thereby removed, whence the oxime was apparently also a dicarboxylic acid.

From the major part of the solution the Cu salt was prepared, by addition of 10 % $CuSO_4$ and alcohol. The precipitated Cu salt was dried at 50°. 20 mg. of green-coloured salt were obtained.

For comparison the Cu salt was prepared in the same way from synthetic oximinosuccinic acid. Both salts were similar in appearance.

Analyses: Cu was determined according to Abderhladen & Schnitzler [1927].

Cu salt prepared from the extract gave on analysis 31·6 % Cu.

Cu salt prepared from oximinosuccinic acid gave on analysis 31·1 % Cu.

The analytical data are in very good agreement. The Cu salt of the oximinosuccinic acid contains theoretically 30·5 % Cu. Low Kjeldahl N-values were

found for both the synthetic Cu salt and that of oximinosuccinic acid isolated from the extract owing to the fact that only a small part of the oxime-N is determined by this method. Both the Cu salts contained oxime-N as determined according to Endres [1935], but the values were in both cases too low owing to the fact that oxime-N is partly oxidized in the presence of Cu. In the synthetic Cu salt 66 % and in the isolated Cu salt 79·6 % of the theoretical amount of oxime-N were found.

Characterization of oxime through its reduction to aspartic acid. Ether solution containing 2 mg. oxime-N, prepared from pea cultures as described above, was evaporated and the residue dissolved in 10 ml. of methyl alcohol. A small amount of finely divided platinum was prepared, and saturated with hydrogen in the cold hydrogenation apparatus of Hückel. The methyl alcoholic solution of the oxime was added and hydrogenated. The hydrogen uptake was 60 ml. in 3 hr. The solution was filtered and evaporated to dryness, the residue dissolved in water and extracted thoroughly with ether in a percolator to remove N-free substances such as succinic acid formed from fumaric acid (cf. below). The aqueous solution was evaporated to a small volume and boiled with freshly precipitated $Cu(OH)_2$. It was filtered hot and kept in the ice-box. A green-coloured Cu salt separated which, after drying in a desiccator, weighed 10 mg. and gave on analysis 23·20 % Cu and 5·66 % N. Cu-aspartate requires 23·06 % Cu and 5·08 % N. Since aspartic acid can be formed on reduction only from oximinosuccinic acid, this must have been the oxime isolated with ether from the cellulose culture of peas.

N-free C-compounds in the extract

N-free dicarboxylic acids. After reduction of the oxime the N-free residue left in the ether was dissolved in water and precipitated with $AgNO_3$, yielding the *Ag salt of succinic acid.* The great excess of hydrogen taken up in the reduction had therefore been used in the reduction of fumaric acid to succinic acid.

Fumaric acid could be isolated with ether from an extract of sand culture of peas, which was kept until all the oxime was decomposed. The amount of fumaric acid was small, 25 mg. of the crude substance being obtained from an extract which contained 120 mg. N. It is possible that in addition to fumaric acid some malic and succinic acids were present in the extract, but these were not investigated more closely.

Amount of aspartic acid among the excretion products at different stages of growth

When sand was washed with water to isolate the excreted N-compounds, it was found that these were very difficult to remove. The thorough extraction, outlined above, with cold water slightly acidified with H_2SO_4 (*p*H 3–4) usually led to the recovery of about 80 % of the excreted N-compounds in the solution. The possibility therefore existed that the remaining 20 % of the N was to be found in compounds other than those in the extract. This fact deserved full attention when theoretical conclusions were drawn.

We therefore tried to transfer all the N-compounds from the medium to the aqueous solution. This was easily attained when finely divided cellulose was used as a medium. Already with two washings all excreted N was recovered from the cellulose in the aqueous solution. The excretion was, however, lower in cellulose cultures than in sand cultures, varying usually from 10 to 20 % of the total fixed N, so that in order to obtain the same quantity of N for investigation much more cellulose cultures would have been required than sand cultures.

Besides, since quartz sand was mostly used as a medium in our experiments, we tried to study as completely as possible the particular N-compounds excreted in such media.

In continuing the extraction experiments we could ascertain that the extraction was generally the more complete the earlier the peas were harvested. Using for extraction water which was acidified with HCl to *p*H 3–4 we succeeded in recovering from young pea cultures, harvested before flowering, practically all the excreted N in the aqueous solution, as shown by the following experiment (Table IV).

Table IV

3 l. Woulff's bottles; 4·8 kg. dry quartz sand and 2 l. N-free nutrient solution in each bottle. 2 Torsdag peas in each bottle, inoculated with strain HX. Period of growth 17 Sept.–22 Oct. 1937. Plants were still not in flower at the end of the experiment. Roots were removed and the sand from all bottles was mixed together. 8 kg. of sand were extracted at a time with cold water in the apparatus shown in Fig. 1. Water was acidified with HCl to *p*H 3–4. All aqueous extracts were evaporated together *in vacuo* to 200 ml.

						In the aqueous extract		
No. of exp.	Dry wt. of plants g.	N in plants mg.*	N excreted in sand, mg.*	Total fixed N, mg.	Extent of excretion	N, mg.	N, % of the excreted N in sand	Aspartic acid-N % of total excreted N
1	1·116	14·5	16·3	30·8	52·9			
2	0·940	14·1	5·0	15·0	33·3			
3	1·115	11·5	16·3	27·8	58·6			
4	0·936	10·4	10·1	20·5	49·3	64·6	99·8	74·7
5	1·115	14·9	5·0	20·0	25·0			
6	1·382	18·2	5·0	23·2	21·5			
7	0·699	13·8	7·0	10·8	64·8			

* Control subtracted.

In this experiment 74·7 % of excreted N was *l*-aspartic acid-N. This amount was appreciably higher than in previous experiments. The difference is due, as will be seen from the data below, to the fact that aspartic acid decreases while β-alanine increases at later stages of growth, a result which is to be expected. Since in the earlier experiments the plants had been in flower, the amount of aspartic acid was already smaller.

In the extract obtained from the experiments presented in Table IV the same qualitative amino-acid analysis was carried out as described earlier in connexion with extract II; the results were identical. With the exception of aspartic acid the extract thus contained no other amino-acids normally present in proteins. β-Alanine could be detected in the extract, since acetaldehyde was formed in the ninhydrin reaction after removal of aspartic acid. In addition, the extract contained some nitrite- and oxime-N:

	% of total N
N precipitated according to Foreman (*l*-aspartic acid-N)	74·7
N precipitated with phosphotungstic acid (β-alanine, calculated)	23
Oxime- and nitrite-N	2

On the basis of this result, the root nodules would not excrete other N-compounds than those found in our investigations, unless the phosphotungstic acid fraction contains some unknown amino-acid in addition to β-alanine. The large amount of aspartic acid in the extract is obviously due to the fact that the cultures were harvested at a very early stage, before flowering. The following series of experiments (Table V), in which the cultures were harvested at different

Table V

The experiment was arranged in the same manner as that in Table IV.

Period of growth	Stage of growth	Dry wt. of plants, g.	N in plants, mg.*	N excreted in sand mg.*	Tota fixed N, mg.	Extent of excretion	In the aqueous extract: N, mg.	In the aqueous extract: N, % of the excreted N in sand	In the aqueous extract: Aspartic acid-N, % of the N in the extract
30. iii.–24. iv. 37	Before flowering	1·319	28·0	9·1	37·1	24·5	28·8	95·1	63·2
		1·648	44·3	13·0	56·3	23·1			
		1·449	33·3	8·2	41·5	19·7			
30. iii.–3. v. 37	At start of flowering	3·456	105·4	35·4	140·8	25·2	118·3	90·9	51·7
		2·947	79·9	51·8	131·7	39·3			
		4·011	118·6	43·7	162·3	27·0			
30. iii.–13. v. 37	In full bloom	3·438	112·1	16·3	128·4	12·8	56·1	87·2	47·0
		3·449	104·0	20·6	124·6	16·5			
		4·460	122·9	27·4	150·3	18·2			
30. iii.–28. v. 37	Pods developed	7·904	167·2	7·7	174·9	4·4	33·1	85·0	35·7
		8·453	159·9	20·2	180·1	11·2			
		8·382	204·4	11·0	215·8	5·1			

* N of the uninoculated controls (14·0 mg.) subtracted.

times, shows clearly that the percentage of aspartic acid-N in the excreted N decreases while β-alanine increases towards the end of the growth, as is to be expected.

This experiment illustrates clearly that the aspartic acid content is higher in younger cultures than in older ones. The experiment also shows that during later stages of growth the pea is able to absorb from the medium through its roots the N-compounds which have been excreted at the earlier stages. In this connexion aspartic acid is of major importance; according to our findings it is an excellent N-source for the legumes.

It is interesting that in older cultures of inoculated peas oxime-N is no longer found.

In 1935–36 we determined the excretion products regularly in pea cultures harvested at flowering stage and obtained extracts which contained about 50% aspartic acid-N. The above data show, however, that early in growth *l*-aspartic acid is the main excretion product, and that it is later partly replaced by β-alanine, so that the amount of *l*-aspartic acid decreases continuously with the age of the cultures. In associated cultures of inoculated peas and non-legumes e.g. barley (sterile system) the amount of aspartic acid is generally higher than in cultures of peas alone, since the non-legume cannot utilize aspartic acid appreciably but can use β-alanine.

Discussion

In the medium of inoculated peas grown in a sterile culture system N-compounds appear immediately after nodule formation. The N-compounds have been shown to be excreted from the root nodules and not from the roots. Their amount may increase so much that the major part of the total fixed N is diffused from the root nodules to the medium and the host plant can utilize only a small part of the fixed N. Such being the case, the chemical nature of the excreted N-compounds is of especial interest from the standpoint of the mechanism of N-fixation.

The excreted N is chiefly amino-N. In young pea cultures the major part of the excreted N is accounted for as *l*-aspartic acid. In addition to aspartic acid

also an amino-acid precipitable by phosphotungstic acid has been found among the excreted N-compounds and has been shown to be β-alanine, which is formed from *l*-aspartic acid by the legume bacteria. Other amino-acids have not been found in the medium of inoculated peas. In addition to amino-N the medium contains some >C:NOH-N and in most cases also some NO_2-N; their total amount together is generally 1–2 % of the total excreted N. The nitrite-N is formed from oxime-N, the oxime in question being oximinosuccinic acid.

The excreted N-compounds and their mutual relations appear from the following:

The excretion products of the root nodules

NO_2 Appears chiefly during extraction and concentration

NOH ‖ $HO_2C.C.CH_2.CO_2H$	→	NH_2 | $HO_2C.CH.CH_2.CO_2H$
Oximinosuccinic acid Generally 1–2 % of the excreted N		*l*-Aspartic acid Varying amounts depending on decarboxylation. In young cultures up to 75 % of the excreted N, in older ones only 20–30 %

NH_2 | $CH_2.CH_2.CO_2H$		
β-Alanine Amounts depend on the decarboxylation of aspartic acid. Therefore more abundantly found in old than in young cultures		

l-Aspartic acid-N and β-alanine-N form together over 90 % of the total excreted N

Taking into account the often exceptionally high rate of excretion as well as the chemical composition of the excreted N-compounds and their mutual relations, the excreted compounds must be regarded as the products of N-fixation. As β-alanine is a secondary decomposition product of aspartic acid, the actual percentage of aspartic acid-N in excreted N is over 90 %. On the other hand, as the ratio of excreted N to total fixed N may rise to 80 %, it can be said that approximately 75 % of the total fixed N may be excreted in the form of *l*-aspartic acid. Thus it is impossible that the excretion products of the root nodules are products of decomposition of bacterial proteins, since the content of aspartic acid in proteins is far from such amounts, being generally below 10 %. According to our determination the N in root nodules contains about 10 % aspartic acid-N. In addition the legumes use aspartic acid excellently as their N-source and on the basis of the facts hitherto known it seems likely that the legumes receive their N-nutrition from the root nodules actually in the form of aspartic acid. Thus the percentage of aspartic acid-N in the total fixed N would rise close to 100 %. *The fixation of atmospheric N in the root nodules thus leads to* l-*aspartic acid, which consequently is a primary fundamental amino-acid.*

The mechanism of the formation of aspartic acid is explained by the isolation of oximinosuccinic acid. This oxime, which is easily reduced to aspartic acid, is obviously the precursor of aspartic acid. Oximinosuccinic acid is formed from hydroxylamine and oxaloacetic acid. Even in extremely dilute solutions oxaloacetic acid and hydroxylamine react instantaneously at slightly acid or neutral reaction so that hydroxylamine cannot be detected in a solution which contains an excess of oxaloacetic acid. No other carbonyl compounds investigated by us react so vigorously with hydroxylamine as does oxaloacetic acid. Oximinosuccinic acid must therefore be formed in the root nodules from hydroxylamine and oxaloacetic acid. Experimentally we have been able to show that oxaloacetic acid is found in leguminous plants; the host plant thus supplies the acceptor for hydroxylamine. The fact that hydroxylamine has not been found among the excretion products of root nodules is easily explicable, owing to its rapid

reaction with oxaloacetic acid. On the basis of the experimental facts the course of biological N-fixation occurring in the leguminous root nodules is as follows:

$$\left.\begin{array}{l} N_2 \rightarrow ? \rightarrow \underset{\text{Hydroxylamine}}{NH_2OH} \\ C_6H_{12}O_6 \rightarrow \underset{\text{Oxaloacetic acid}}{HO_2C.CO.CH_2.CO_2H} \end{array}\right\} \rightarrow \underset{\text{Oximinosuccinic acid}}{HO_2C.C(NOH).CH_2.CO_2H} \rightarrow \underset{\text{l-Aspartic acid}}{HO_2C.CH(NH_2).CH_2.CO_2H}$$

This course of the reaction is in agreement with all the facts hitherto known; the results cannot be interpreted otherwise on the basis of the present knowledge. How hydroxylamine is formed from atmospheric nitrogen has not yet been experimentally proved. It can be assumed hypothetically that a di-imide is formed as a primary reduction product ($N_2 \rightarrow HN{=}NH$) which then forms hydroxylamine by addition of two water molecules. The reduction of the nitrogen molecule, "N-fixation" in a stricter sense, requires obviously a specific enzyme, which is found in the N-fixing bacteria. The reaction of hydroxylamine with oxaloacetic acid is a non-enzymic process. On the other hand, the reduction of oximinosuccinic acid to *l*-aspartic acid is an enzymic reaction. It has not yet been discovered from which compounds in the plant the hydrogen required for the reduction is transferred to the substrate to be reduced, and which enzymes act in the reduction process.

On the basis of the above facts N-fixation requires the presence of oxaloacetic acid. The important role of this C_4-dicarboxylic acid in N-fixation offers an explanation of the symbiosis of legumes and intranodular bacteria, which has hitherto remained mysterious. It has been possible to show synthetically the decisive role of oxaloacetic acid in N-fixation and thus to throw more light on our idea of the reaction mechanism of N-fixation. Excised root nodules do not fix nitrogen in aqueous solution and even in solutions containing glucose N-fixation is either nil or very slight, but in a neutral solution of oxaloacetic acid a vigorous N-fixation occurs already in a few hours [Virtanen & Laine 1937]; simultaneously oxime-N and amino-N appear in the solution. In a series of 25 experiments distinct N-fixation has been accomplished in all the experiments with excised root nodules in presence of oxaloacetic acid. We are going to describe these experiments more closely in another communication.

Aspartic acid is, according to our results, a primary amino-acid formed in the N-fixation. Other amino-acids must therefore be formed from this fundamental amino-acid. In the pea plant the reaction:

$$HO_2C.CH_2.CH(NH_2).CO_2H + CH_3.CO.CO_2H \rightleftarrows HO_2C.CH_2.CO.CO_2H + CH_3.CH(NH_2).CO_2H$$

takes place rapidly, so that the formation of other amino-acids from aspartic acid is easily understood [Virtanen & Laine, 1938, 2]. In plants the reamination (*Umaminierung*) occurs at least partly in the same manner as, according to Braunstein & Kritzman [1937], it does in animal tissues. It is surprising that no glutamic acid is formed in N-fixation by root nodules, although glutamic acid occupies a special position in amino-acid synthesis. In v. Euler's laboratory [Adler *et al.* 1938] several important investigations have been carried out during the last year on the dehydrogenation of glutamic acid and on the reamination taking place thereby, which according to these findings is caused in micro-organisms and plants by a specific glutamic acid dehydrogenase.

The reaction mechanism of the N-fixation occurring in free-living *Azotobacter* is obviously similar to that occurring in the intranodular legume bacteria. The

excretion of N-compounds by *Azotobacter* is, however, so slight that it would be impossible to conclude anything from it, if the results attained with legume bacteria did not justify the conclusions by analogy. The great difference in the excretion by *Azotobacter* and by intranodular legume bacteria is obviously due to the fact that the former uses the products of N-fixation largely for the formation of its cell protein, while the latter excrete the major part of them. We shall give later a detailed report of our experiments with *Azotobacter*.

Summary

The N-compounds excreted from the root nodules of leguminous plants have been isolated and characterized.

Over 90% of the excreted N is amino-N. In addition, 1–2% oxime-N and some nitrite-N is found.

The major part of the amino-N is present as *l-aspartic acid*, if peas are harvested at a young stage, long before flowering. In ageing cultures the amount of aspartic acid decreases.

The other amino-acid found among the excretion products is *β-alanine* which is slowly formed from *l*-aspartic acid by the legume bacteria. Its formation explains the decrease of aspartic acid in ageing cultures.

The oxime appearing among the excretion products is *oximinosuccinic acid*; nitrite-N is formed from this oxime.

Some *fumaric acid* is detected as an N-free excretion product.

The often exceptionally high amount of excreted N (60–80% of the total fixed N) and the special nature of the excreted N-compounds prove that these N-compounds are products of N-fixation. An idea has thus been obtained, on the basis of the excreted N-compounds, of the mechanism of biological N-fixation.

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LIV. THE ULTRAVIOLET ABSORPTION OF SHEEP THYROGLOBULIN

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Analysis of the ultraviolet absorption curve of thyroglobulin in terms of the absorption of its iodine-containing components thyroxine and diiodotyrosine leads to interesting results. The absorption spectra of these amino-acids have previously been measured by Hicks [1925], Abderhalden [1927] and Heidt [1936]. In Fig. 1 our own results are given. The measurements were made on alkaline solutions (0·02 *N* NaOH) and acid solutions (diiodotyrosine in 0·1 *N* HCl; thyroxine in 0·014 *N* HCl). At the *p*H of the alkaline solutions ($pH \geqq pK+2$) the phenolic hydroxyl, the ionization of which governs the change of absorption spectrum from the acid form to the alkaline form [Stenström & Reinhardt, 1925, 2], is almost completely ionized whereas in the acid solutions ($pH \leq pK-2$) it is almost completely unionized. For diiodotyrosine

$$pK = 6{\cdot}2.$$

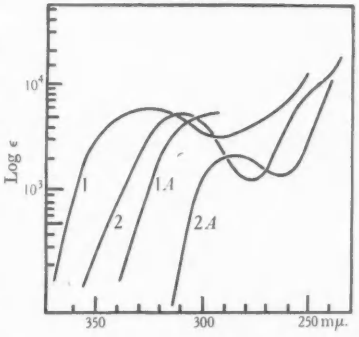

Fig. 1. Molecular extinction coefficients of: 1, thyroxine in 0·02 *N* NaOH; 1*A*, thyroxine in 0·014 *N* HCl; 2, diiodotyrosine in 0·02 *N* NaOH; 2*A*, diiodotyrosine in 0·1 *N* HCl.

In these absorption curves no "fine structure" (vibrational structure) is present. We cannot, therefore, expect to be able to detect the presence of thyroxine and diiodotyrosine in thyroglobulin by characteristic peaks in the thyroglobulin curve. This conclusion is confirmed by the smoothness of the absorption curves of thyroglobulin at *p*H 8·0 and *p*H 1·3 (Fig. 2). The curves show a single maximum at 280 mμ—the well-known protein band due to tyrosine and tryptophan. No maximum can be distinguished in the region of the alkaline absorption bands of thyroxine and diiodotyrosine from 350 to 300 mμ.

Two methods of quantitative analysis are possible. First, one can compare the thyroglobulin absorption curve at *p*H 8·0 with that of serum globulin at *p*H 8·0 (curve 2, Fig. 2). This method is open to objections, e.g. the difference between serum globulin and the globulin in thyroglobulin. A second method has proved to be more satisfactory. Here the different compounds are not characterized in the usual way by an absorption curve which represents the (molecular) extinction coefficient as a function of the wave-length, but by a curve which represents the so-called differential (molecular) extinction coefficient as a function of the wave-length.

The differential extinction coefficient δ at a given wave-length is defined as the difference between the alkaline extinction coefficient K (at $p\mathrm{H} \geqq pK+2$) and the acid extinction coefficient K' (at $p\mathrm{H} \leqq pK-2$). In a similar way the differential molecular extinction coefficient δ_ϵ is defined as the difference between the alkaline molecular extinction coefficient ϵ and the acid molecular extinction coefficient ϵ'.

The advantage of the use of δ instead of K or K' lies in the fact that δ is zero for all compounds which show no shift in the absorption spectrum in the given pH range.

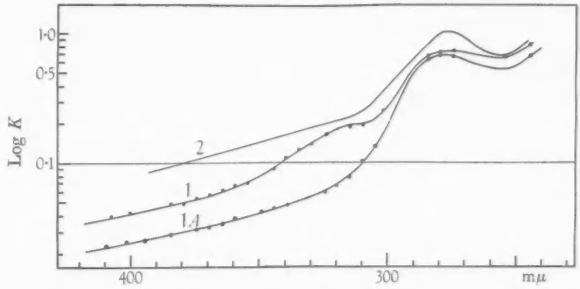

Fig. 2. 0·7% thyroglobulin solution at pH 8·0 (1) and at pH 1·3 (1*A*). 1·0% sheep serum globulin (2).

By means of the δ curves it has been possible to analyse the thyroglobulin curves in the region 400–300 mμ. An extension of the analysis to wave-lengths below 300 mμ failed because of masking by the protein bands. In Fig. 3 are

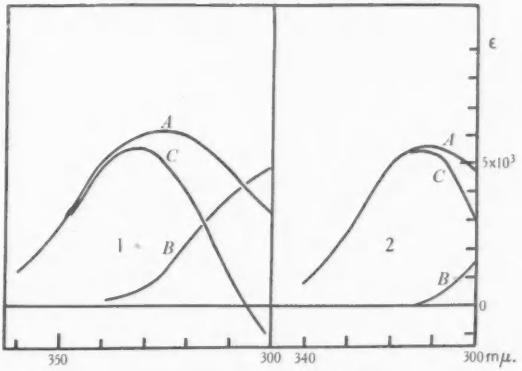

Fig. 3. 1, Molecular extinction of thyroxine; 2, molecular extinction of diiodotyrosine; *A*, in alkali; *B*, in acid; *C*, molecular differential extinction.

represented the δ_ϵ, ϵ and ϵ' curves of thyroxine (1) and diiodotyrosine (2). The values of the molecular (differential) extinction coefficients are given in Table I.

As may be noticed, the δ_ϵ bands are more sharply defined than the ϵ bands and for this reason they are more apt to characterize the compounds. The following is a description of the way in which the δ curve of the iodine compounds

Table I

Thyroxine							
λ (mμ)	360	350	340	330	320	310	300
$10^{-3}\,\epsilon$	1·1	2·6	5·0	6·1	6·1	4·9	3·4
$10^{-3}\,\epsilon'$	—	—	0·1	0·4	2·1	3·9	5·0
$10^{-3}\,\delta_\epsilon$	1·1	2·6	4·9	5·7	4·0	1·0	−1·6
Diiodotyrosine							
$10^{-3}\,\epsilon$	—	—	0·8	2·4	4·9	5·6	4·5
$10^{-3}\,\epsilon'$	—	—	—	—	—	0·3	1·5
$10^{-3}\,\delta_\epsilon$	—	—	0·8	2·4	4·9	5·3	3·0

in thyroglobulin can be derived from the absorption curves of thyroglobulin at *p*H 8 and *p*H 1·3.

In the region from 400 to 300 mμ the absorption K_t of thyroglobulin is determined by the following factors (Fig. 2, curves I and I*A*):

(*a*) Scattering of light: K_s (present from 400 to 300 mμ).

(*b*) Absorption of the iodine components K_i (present from 370 to 300 mμ).

(*c*) Absorption of the globulin K_g (present from 320 to 300 mμ).

Each of these quantities will be discussed briefly.

K_s is caused by the presence of larger protein particles in the solution which cannot be centrifuged down even at 15,000 rev./min. The properties of this scattering have been studied in solutions of serum globulin (Fig. 2, curve 2); there is no reason why the scattering of serum globulin should differ much from the scattering of thyroglobulin. The serum globulin used was prepared in a way similar to that used for the preparation of thyroglobulin.

The scattering was found to be proportional to $1/\lambda^4$. Thus the scattering could be described by the Rayleigh-Jeans formula for the scattering of particles which are small compared with the wave-length of the incident light.

K_i occurs below 380 mμ in the thyroglobulin curve. Above this wave-length the thyroglobulin curve is similar to the serum globulin absorption curve (Fig. 2, extinction coefficient proportional to $1/\lambda^4$). Below this wave-length the thyroglobulin curve increases more rapidly than the serum globulin curve owing to the occurrence of K_i in the thyroglobulin curve. K_g is only of importance below 320 mμ [Gróh & Hanák, 1930]. The value of K_g is the same at *p*H 8 and at *p*H 1·3. K_g increases first at *p*H values above 10 [Stenström & Reinhardt, 1925, 1]. Now we can write that at *p*H 8: $K_t = K_s + K_i + K_g$ and at *p*H 1·3: $K_t' = K_s' + K_i' + K_g'$. Because $K_g = K_g'$ we can deduce that

$$K_i - K_i' = (K_t - K_t') - (K_s - K_s').$$

The left-hand member of this equation represents the differential absorption δ_i of the iodine compounds. The values of K_t and K_t' present in the right-hand member are determined experimentally.

K_s and K_s' are only known at wave-lengths above 370 mμ (here K_i and K_g are both zero, the absorption is only due to K_s). At those wave-lengths $K_s = K_t$ and $K_s' = K_t'$. By application of the Rayleigh-Jeans formula, we can find the values of $K_s - K_s'$ below 370 mμ by extrapolation. If these values are calculated by extrapolation of the value at 380 mμ, which value is denoted by $(K_t - K_t')_{380}$, the formula for $K_i - K_i'$ or δ_i becomes

$$\delta_i = (K_t - K_t') - (K_t - K_t')_{380} \times (\lambda/380)^4.$$

In the deduction of this formula we have assumed that *p*H 8 and *p*H 1·3 lie outside the interval from $pK - 2$ to $pK + 2$. That the assumption is sound was verified by the measurement of K_i at different *p*H values (*p*H 8·0, 6·8, 6·0) and calculation of the values of $K_i - K_i'$. From these data a value between 5 and 6

was deduced for the pK value of the iodine compounds in thyroglobulin by means of a well-known formula from the theory of dissociation. Therefore pH 8 and 1·3 lie on either side of $pK-2$ and $pK+2$.

By means of equation (1) the δ curve of the iodine compounds present in thyroglobulin was deduced from the absorption curves of solutions of sheep thyroglobulin (for the preparation of solutions see below under (*b*)).

The shape of these δ curves agreed fairly well with the curves which could be calculated if half of the iodine in the thyroglobulin solution was attributed to thyroxine, the other half to diiodotyrosine. Moreover, the values of the total iodine per ml. of solution which were obtained from the δ curves did not differ much from the values which were obtained by direct chemical analysis:

	1	2	3	4	5	6
Iodine per ml. of solution: δ curves	36	38	36	45	47	49
Iodine per ml. of solution: chemical analysis	38	42	39	47	49	52

1, 2, *Elityran* (a liquid thyroglobulin preparation of I.G. Farbenindustrie) after extraction with ether; 3, own preparation of thyroglobulin; 4, 5, 6, thyroglobulin I.G. Farbenindustrie.

The δ curves of the iodine compounds in thyroglobulin which were measured for two solutions (of different samples of thyroglobulin) are reproduced in Fig. 4, curves *A* and *B*. The error in the δ values is 3% (error in extinction 1·5%). Accidentally the δ curves coincide at 320 mμ. Now these curves must be compared with the δ curves which can be calculated for a thyroxine-diiodotyrosine mixture containing two diiodotyrosine molecules to one thyroxine molecule (iodine equally distributed over thyroxine and diiodotyrosine). This calculated curve is represented in Fig. 4, curve 2. Besides the condition mentioned (thyroxine-iodine = diiodotyrosine-iodine), the total quantities of thyroxine and diiodotyrosine are chosen in such a way that the calculated curve coincides at 320 mμ with the experimental curves.

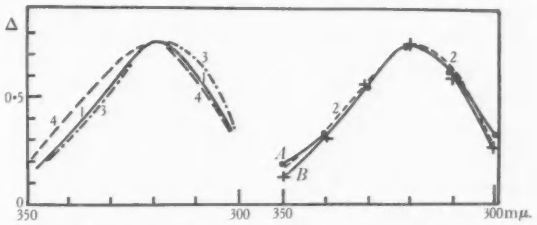

Fig. 4. Experimental δ curves (*A* and *B*). Mean values of *A* and *B* (curve 1). Theoretical curve based on ratio of thyroxine-iodine to diiodotyrosine-iodine of 1 : 1 (curve 2); 4 : 6 (curve 3); 6 : 4 (curve 4).

It may be seen that the agreement between calculated and experimental curves is satisfactory. If we compare the total iodine per ml. of solution, which can be deduced from the curve, with the chemical values (as has been done above for six other solutions) we find 55 μg. per ml. of solution from the δ curve whereas the chemical values are 55 μg. and 51 μg.

In curves 3 and 4 (Fig. 4) the lack of agreement between calculated and experimental δ curves is demonstrated if we base the calculation on 40% thyroxine-iodine (curve 3) and on 60% thyroxine-iodine (curve 4). Under these conditions a considerable difference in shape between the calculated and experimental curves (curve 1) is shown. Therefore the value of the diiodotyrosine-

iodine/thyroxine-iodine ratio may be determined with fair accuracy by means of an analysis of the δ curve. This ratio cannot differ much from unity in the seven samples of sheep thyroglobulin which have been studied here.

It seems interesting to check whether the same value for this ratio is valid for other kinds of thyroglobulin (ox, hog). As a first experiment the absorption of a solution of ox thyroglobulin was examined (0·7 % solution of ox thyroglobulin, I.G. Farbenindustrie).

The experimental δ curve agreed with the δ curve of a diiodotyrosine-thyroxine mixture which contains 50 % thyroxine-iodine. The δ curve corresponded to 21 μg. iodine per ml.; the chemical analysis yielded 24 μg. iodine per ml.

These data do not agree with experiments of Leland & Foster [1932], or of Abelin [1932] who found a mean value of about 0·4 for the ratio diiodotyrosine-iodine to thyroxine-iodine in thyroglobulin. Our results seem to agree better with determinations of thyroxine-iodine which have been carried out by Harington & Randall [1929], and Rotter & Mecz [1932]. Rotter & Mecz found a mean value of 1 for the diiodotyrosine-iodine/thyroxine-iodine ratio (19 different thyroglobulin preparations). However, their values vary from 1·4 to 0·4. Our values do not show such variations. This may be explained by the relatively small number of samples (8) which have been studied by us, but will be verified in further experiments.

Here the disadvantage of chemical methods used for the determination of the thyroxine content of thyroglobulin, i.e. changes of the chemical structure, is avoided. On the other hand, the absorption method can only be used for thyroid preparations which yield a clear solution at *p*H 8 and *p*H 1·3. The extinction coefficient at 400 mμ is below 0·7 if iodine concentration is 20 μg. per ml. of solution.

We may conclude that this spectrographic analysis offers strong support for the conception that —C.$C_6H_2I_2$.O.$C_6H_2I_2$OH groups (chromophoric group of thyroxine) and —C.$C_6H_2I_2$OH groups (chromophoric group of diiodotyrosine) exist in the thyroglobulin molecule. The absorption is mainly due to these chromophoric groups and not to the whole molecule; hence we must not deduce the occurrence of thyroxine and diiodotyrosine as such in the thyroglobulin molecule; changes in the side chains of these iodine compounds remain possible.

Some attention may be paid to the small difference in shape between the experimental and calculated curves. The accuracy of most of our measurements of the δ curve have a mean error of 6 %. However, the experimental curves *A*, *B* of Fig. 4 were determined with the utmost care (see below under *d*). Therefore we must accept the reality of the small differences between these curves and the theoretical curve (curve 2, Fig. 4).

Summary

1. An analysis of the thyroglobulin absorption curve in the region from 400 to 300 mμ is given. In this analysis differential extinction coefficients have been used. This quantity is defined as the difference between the extinction of the solution if practically all the absorbing molecules have the alkaline form (*p*H above $pK+2$) and the extinction of the solution if practically all the absorbing molecules occur in the acid molecule-form (acid absorption, *p*H below $pK-2$).

2. The differential absorption curve of the iodine compounds, as deduced from the absorption curves of (sheep) thyroglobulin at *p*H 8 and 1·3, gives strong evidence for the presence of the —C.$C_6H_2I_2$OH group (chromophoric group of diiodotyrosine) and the —C.$C_6H_2I_2$.$C_6H_2I_2$OH group (chromophoric group of

thyroxine) in the thyroglobulin molecule; two of the former groups occur to one of the latter groups.

These experiments were suggested by Prof. I. Snapper, to whom the author wishes to express his sincere thanks. He is also indebted to the laboratory staff, especially to Dr A. Grünbaum. Expenses were met by a grant from the Rockefeller Foundation.

Appendix

(*a*) Determination of the acid absorption of thyroxine. A first attempt to determine the acid thyroxine absorption directly failed (absorption tube of 120 cm. filled with saturated thyroxine solution in 0·1 *N* HCl). Therefore we made use of the adsorption of thyroxine on to a substance which remained soluble at *p*H 1·3.

Dr W. M. Bendien in this laboratory has shown that such a protective colloid is present in serum (horse, human, sheep). He found that thyroxine which is perfectly ultrafiltrable in its solution in 0·02 *N* NaOH becomes non-ultrafiltrable if dissolved in human serum: evidently thyroxine is adsorbed by one of the constituents of the serum (? globulin). With this fact in mind it was easy to get (adsorbed) thyroxine into acid solution.

2 ml. horse serum are mixed with 8 ml. of a 0·066% thyroxine solution (0·02 *N* NaOH). Then 0·3 ml. of *N* HCl is added. In this way the solution is brought to *p*H 1·8 without any precipitation of thyroxine. By subtracting the absorption of a mixture containing 2 ml. horse serum, 8 ml. of 0·02 *N* NaOH and 0·3 ml. of *N* HCl from the absorption of the former solution, the absorption of the thyroxine in acid solution was obtained. Adsorbed thyroxine and free thyroxine show the same absorption, as could be verified in alkaline solution; no appreciable difference could be found between the absorption of a thyroxine solution in 0·02 *N* NaOH and the absorption of a thyroxine solution in alkaline serum.

(*b*) Thyroglobulin is not easily dissolved if directly treated with acid. In order to obtain an acid solution, the thyroglobulin must be dissolved first in an alkaline solvent. The borate buffer solution of *p*H 8 may not be used as a solvent: if this solution is made acid by adding *N* HCl solution, a small part of the thyroglobulin is precipitated. This is not the case if the thyroglobulin is dissolved in 0·02 *N* NaOH. Thus thyroglobulin solutions at *p*H 8 and 1·3 which may be used for the absorption analysis are obtained by first preparing a solution of thyroglobulin in 0·02 *N* NaOH (about 8 mg. thyroglobulin per ml.). One part of this solution is acidified by adding quickly *N* HCl till the *p*H reaches 1·3, whereas to the other part concentrated buffer-solution is added to bring the *p*H to 8.

(*c*) The photographic method which was used for the determination of the absorption curves (except those reproduced in curves *A* and *B*, Fig. 4) has been described previously [Ginsel, 1936]. Because this method is based on the use of density marks, the absorption coefficients can be determined at any given wavelength. Its simplicity is due to the fact that only those transparencies of the photographic plate are used which correspond to the straight part of the transparency curve (transparency is intensity of light transmitted by exposed area divided by intensity of light transmitted by unexposed area; transparency curve is transparency plotted against log of relative intensity).

It is stated [Ginsel, 1936] that transparencies between 0·35 and 0·65 correspond to the straight part of the transparency curve. After a longer experience with

Ilford Auto filter plates (400 H and D) we have found however that small deviations from straightness may occur sometimes in this transparency region. The only part of the transparency curve which shows perfect straightness in all the cases is located between transparencies 0·45 and 0·65.

If we are limited to the use of this transparency region, it is only possible to obtain a complete absorption curve if the thicknesses of the liquid layers increase by a factor not greater than 1·7. Unfortunately our tubes increase by a factor 2. Therefore we could not avoid the use of transparencies lying outside the range 0·45–0·65. In this way small errors (below 3 %) may have been introduced in most of our absorption curves.

In the determination of curves *A* and *B*, Fig. 4, we did not use the simple method developed by us but reverted to the actual use of density curves, in order to reduce sources of errors. The absorption coefficients were deduced from the densities on the photographic plate in a way similar to the well-known procedure used in photographic intensity measurements.

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LV. AN ENZYME OF THE TUBERCLE BACILLUS[1]

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The possible importance of bacterial enzymes in the diseases of man has not received the attention it deserves. Particularly in a chronic disease such as tuberculosis the bacterial enzymes, disseminated slowly but with cumulative effect, may well play a decisive role. The study which follows deals with an oxidizing enzyme produced by the tubercle bacillus.

The findings of four groups of workers are in direct conflict in regard to the presence of an indophenol oxidase in the tubercle bacillus. Gordon & McLeod [1928], using *p*-aminodimethylaniline (*p*-dimethylphenylenediamine) alone, reported a 1 plus result for the tubercle bacillus on a scale where the *meningococcus* was 4 plus, confirming the 1 plus result of Schultze [1910] and Kramer [1912] who used the diamine in combination with α-naphthol. But Terada & Nozaki [1937], in a study concerning human and rat lepra bacilli and the other acid-fast organisms, using the diamine alone as did Gordon & McLeod, found no indophenol oxidase in either the pathogenic or non-pathogenic acid-fast bacilli, while their controls of *B. subtilis* and *meningococcus* gave the proper positive results. Kawabata [1934] also reported negative results for indophenol oxidase in human, bovine and frog tubercle bacilli.

No reference has been found in the literature to the presence in the tubercle bacillus of a catechol or phenol oxidase, but a catechol oxidase frequently occurs in organisms producing an indophenol oxidase as shown by Happold [1930]. The intensities of the enzymic reactions were not parallel for the two, and one organism (*Staph. albus*) possessed the catechol but not the indophenol oxidase.

A search for the enzyme which is the subject of this paper was undertaken because it appeared that some of the properties of a phenolase or catechol oxidase might explain certain observations made in the laboratory regarding pigmentation in tuberculous tissue, and the reddening and darkening of masses of tubercle bacilli which had been left for some weeks in a phenol solution. The work described is preliminary to a more extended study now under way.

Experimental

Testing of bacillary masses with phenolic substances

The original observation regarding the darkening of masses of bacilli was verified by placing equivalent masses of tubercle bacilli of human type in duplicate bottles of 5% phenol, sterile physiological saline and sterile distilled water; one trio was kept in the dark and the other was exposed to the usual laboratory lighting. The bacillary masses changed colour only in phenol, and the reddening occurred sooner and darkened more in those exposed to the light. In all subsequent tests the materials were accordingly exposed as evenly as possible to light.

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Variation of phenol concentration. Aqueous solutions were used. Ten concentrations, from 0·5 down to 0·1 %, and from 0·05 to 0·01 % respectively, were tested in 5 ml. portions. The human type bacillus (Gary strain) was used; one culture had been recently planted and the other was an old one, suspected of being dead. Comparable crumbs of bacilli (20–40 mg.) were removed from the solid medium and placed in the solutions with a nichrome wire loop.

Reddening of both organisms was observed in 1 day in the 0·5, 0·4 and 0·3 % tubes. Both reddened in 2 days in the 0·2 % phenol. The freshly recovered strain reddened in the 0·1 % tube in 4 days, but not the old culture. None of the 0·05–0·01 % tubes gave any coloration within 2 weeks. For practical purposes, therefore, it would be advisable not to use a lower concentration of phenol for testing than 0·2 %.

An upper limit for the phenol concentration cannot be determined. Homogeneous mixtures of phenol and water cannot be obtained between approximately 10 and 70 % phenol. Human type bacilli (Gary), taken off solid medium, were tested in 90, 80 and 75 % phenol. Swelling and some dispersion of the crumbs occurred, but no darkening within 12 days. Five months later complete solution of the bacillary masses was the only change to be observed.

Phenol in phosphate buffer. KH_2PO_4-NaOH mixtures, made up according to Clark [1925] at *p*H 5·8, 6·0, 6·2, 6·4, 6·6, 7·0 and 7·4, containing 5 % phenol, were used in 5 ml. portions with equal-sized crumbs of human bacilli (Gary) freshly grown on 5 % glycerol broth. In 1 day a faint, shrimp-pink tint was seen on the edges of the crumbs in all the tubes. After 3 days the colour had spread throughout the bacillary masses, that in the *p*H 6·0 tube being the darkest; next day there was a suggestion of dark speckling in this tube. Two months later only a uniformly deep rose colour appeared in all the tubes, somewhat greyer in tinge than the original.

Preliminary trials with substances other than phenol

Orcinol, thymol and p-*benzoquinone* were tested in saturated aqueous solution; *catechol* and *resorcinol* were used in 1 % aqueous solution. Crumbs of the same human type bacilli (Gary), used above for the phenol-phosphate tests, were used here, in 5 ml. of the solutions of which another 5 ml. were kept as a control in which colours developed by autoxidation could be seen.

There was no immediate change in any tube except that containing the quinone, where a deep red-brown developed in the solution around the bacillary masses; in 22 hr. the flakes were deep brown and the solution itself darker than the control. At 22 hr. the catechol solution containing bacilli was pink, the control colourless; the orcinol test mixture was slightly pinker than its control. After 48 hr. the quinone-coloured flakes were very dark, those in the catechol were reddish grey (with the autoxidation control still colourless), and those in orcinol had faint pink edges. After 7 weeks the quinone mixture was dark and very turbid, preventing further description; the bacilli had become brownish black in catechol (while the control was only a weak tea-colour); in orcinol the masses were a rosy, greyish brown, the control a light brownish red. Resorcinol and thymol solutions remained negative throughout, no colour developing with or without bacilli.

In connexion with the colour developed in the *p*-benzoquinone, curiosity prompted the filtration of some of the culture fluid from this same lot of bacilli through a Berkefeld grade N candle, and the testing of the filtrate with quinone. A deep brownish red colour developed instantaneously and became more intense overnight. A portion of sterile glycerol broth of the same batch used for the culture was also tested with quinone and gave qualitatively similar results but

very much more slowly. Presumably substances capable of interaction with quinone had been added to the medium during bacillary growth. It should be noted that the bacilli themselves became darkened in the presence of quinone, and while this might mean further oxidation of quinone by enzymic means, it might mean a localized combination of quinone with bacillary substance, which was dark in colour.

Phenol, catechol, resorcinol, quinol, pyrogallol, hydroxy-quinol (1:2:4-trihydroxybenzene), phloroglucinol, tyrosine, adrenaline

Phosphate buffer at *p*H 6·6 was used to prepare 1% solutions of all the phenol family, and a saturated solution of tyrosine; a 5 ml. portion of each solution was used for the test, and 5 ml. used as a control for observation of colour developing with autoxidation; the adrenaline used was the commercial 1:1000 solution of the hydrochloride, containing preservatives. Crumbs of equal size of human type bacilli (strain Gary), grown on solid medium, were placed in the test solutions.

At this *p*H (6·6), during 44 hr. observation, the phloroglucinol, resorcinol, phenol and tyrosine tests were all negative. Results with the other compounds follow. 1:2:4-Trihydroxybenzene: the control itself darkened too readily to permit observation of the bacilli, but they were already very dark at 8 hr. Pyrogallol: the bacillary mass was orange at 8, very brown at 31 and black at 44 hr. Quinol: a questionable coloration of the mass was noted at 8 hr., which had deepened to a definite rose at 31, and to a dark reddish brown at 44 hr. Catechol: the bacillary mass was brownish at 8 hr., and the brown colour became progressively more intense at 31 and 44 hr. Adrenaline: no colour change had been observed at 44 hr., but in view of the preservatives present (chloretone $2\frac{1}{4}$ grains per ounce, and sodium bisulphite not over 0·1%) the tube was kept for further observation; after 27 days the bacillary mass had become a brownish grey, which was still darker after 6 more days. In the absence of preservatives the adrenaline would be expected to show an activity similar to that of catechol; the concentration of the adrenaline was also only one-tenth that of the catechol.

From the respective colorations of the bacillary masses with passage of time, one would derive the following order of diminishing oxidizability (diminishing reducing power) at *p*H 6·6: 1:2:4-trihydroxybenzene, pyrogallol, catechol, hydroquinone, adrenaline (with preservatives present), and phenol (other long-term experiments with Gary cultures).

Production of dianilino-*o*-benzoquinone

Because of the several chemical manipulations necessary, the non-virulent type of tubercle bacillus (BCG) was chosen for the first experiment, which followed substantially the preparation of dianilino-*o*-quinones by Pugh & Raper [1927] and by Happold [1930] using enzymes from various sources. Sterile apparatus was used throughout.

A suspension of BCG was prepared by grinding crumbs of bacilli with physiological saline in an agate mortar; the 35 ml. of suspension used for the experiment contained 105 mg. of bacilli. The suspension was added to 100 ml. of Clark's K_2HPO_4-NaOH buffer at *p*H 6·5, containing 1 g. catechol and 2 ml. aniline. No immediate colour developed after mixing, and the preparation was allowed to stand overnight, free access of air being provided through the sterile cotton plug in the flask. After 16 hr. a red precipitate with a somewhat brownish tinge appeared, consisting of just-visible tufts of fine short needles; the liquid

was coloured red also. The flask was aerated for 2 hr. with gentle bubbling, first passing the air through chloroform-water, and a marked increase in the amount of precipitate resulted.

A few drops of the mixture, containing some precipitate, were examined microscopically. Beautiful garnet red bundles of needles were seen among considerable débris; feather and burr forms and single needles were all present. The red crystals in the precipitate were extremely friable; a portion of the mixture was filtered, but the crystals could not be removed from the filter paper without admixture with paper fibres; the filter paper was dried over sulphuric acid. The M.P. of the crude material, still containing bacilli and débris, was 186·5° (uncorr.).

Of all preparations of dianilino-*o*-benzoquinone made subsequently, the one to be described next was the most successful.

Culture fluid with catechol and aniline

The culture fluid (75 ml.) from a 3 months' growth of human type bacillus (Gary) upon 5% glycerol broth was decanted from bacillary sediment after centrifuging. To it was added an equal volume of phosphate buffer at *p*H 6·5, containing 1 g. catechol and 2 ml. aniline.

A faint pink colour developed immediately, rapidly darkening in tint; the reaction mixture was red and a few red needles had already formed in 15 min. In 5 hr. a heavy precipitate had settled, which was increased in bulk on standing overnight; the liquid still contained much red material in suspension. This preparation was not aerated at any time, but absorbed the necessary oxygen at its surface. (Owing to the virulence of this strain, whose bacilli might be present in the precipitate, no chemical manipulation of the product was attempted.)

The control flask, made up of 75 ml. (sterile) of the same lot of glycerol broth on which the culture had been grown, with the same buffer, catechol and aniline, produced a small amount of dirty light brown flocculent material after an hour; this settled out overnight, leaving a clear light brown liquid; no red crystals were seen at any time during the 18 hr. the mixture was under observation. It is therefore clear that the growth of tubercle bacilli in this broth had produced an agent capable of very marked catalysis of the oxidation of catechol, as manifested by the rapid and voluminous formation of the dianilino-derivative of its oxidation product, *o*-benzoquinone.

Since the result of using decanted culture fluid instead of a bacillary suspension was so satisfactory, the next step was to try a fluid which could not be suspected of containing any bacilli whatever.

Berkefeld filtrate from human type bacilli

The culture fluid from a growth of the virulent strain H 37 upon 5% glycerol broth was passed through a grade N Berkefeld candle and tested with the usual proportions of catechol and aniline in phosphate buffer at *p*H 6·5. A very marked catalysis of the oxidation of catechol was observed.

Further possible avenues of approach to a study of bacillus-free preparations of the enzyme were thus opened.

Note. Another preparation of the dianilino-benzoquinone was made, using a sterile Berkefeld filtrate from a culture of the non-virulent BCG. The red crystals, after decanting the reaction mixture, were washed once, centrifuged and spread on a clay plate. The M.P. of this still relatively crude product (air-dried for 3 days) was 190°, with decomposition, which compares favourably with 193°, the value reported by Happold for his most highly purified product.

Effect of bacillary suspension on phenol, guaiacol, orcinol and quinol in presence of aniline

A suspension of washed human type bacilli (Gary) was used for all these experiments. Approximately 50 mg. of bacilli were used for each 50 ml. of phosphate buffer at *p*H 6·5, containing the usual proportions of the phenol in question, and aniline.

None of these phenols formed the red dianilino-*o*-benzoquinone. No visible product appeared in the guaiacol mixture; brownish yellow substances were observed in the phenol, orcinol and hydroquinone mixtures.

The failure to obtain evidence of the formation of an *o*-quinone in the case of orcinol agrees with the conclusion of Happold [1930] concerning the oxidation of that substance, and in the cases of phenol and guaiacol it may perhaps be considered additional evidence that the mode of action of the enzyme under discussion is not that of tyrosinase [Pugh & Raper, 1927].

Old tuberculin, long-dried bacilli

It seemed of interest to investigate the retention of this particular enzymic activity in old tuberculin (which is essentially a concentrated sterile filtrate of culture broth from the human type bacillus), and in a stock of dried human type bacilli (H 37) which had been kept over sulphuric acid for approximately 10 years.

10 ml. of old tuberculin (representing 100 ml. of fresh culture fluid) were mixed with 90 ml. of distilled water and 100 ml. of phosphate buffer at *p*H 6·5, containing the usual 1 g. catechol and 2 ml. aniline. The weight of the dried bacilli used, 0·54 g., was approximately five times that used in the fresh bacillary suspensions previously tested; the bacilli were suspended in 80 ml. buffer at *p*H 6·5, containing 0·75 g. catechol and 1·5 ml. aniline. In this case an autoxidation control of the reagents was kept, since it was expected that observations would continue for a long period. (The control of sterile broth used with the decanted culture fluid-catechol experiment was also available for comparison with the old tuberculin.) After 2 months so much brownish deposit had formed that it was useless to try to weigh the yields of red crystals of the dianilino-ortho-benzoquinone, and only visual comparison was possible.

There appeared to be little difference between control and dried bacilli. Apparently the enzymic activity had been lost during the ageing and drying of the bacilli.

The old tuberculin mixture, however, showed quite definitely a heavier yield than the controls. This particular preparation thus had seemingly retained some of its oxidase activity, but whether this represented the total activity of the old tuberculin after manufacture could only be decided by tests at repeated intervals of storage.

ATTEMPT TO USE METHYLENE BLUE AS HYDROGEN ACCEPTOR IN THE OXIDATION OF CATECHOL

It was thought that if methylene blue could be utilized as hydrogen acceptor in place of molecular oxygen, a quantitative method for estimation of the enzyme could be worked out, using catechol as the substrate and depending on the rate of decoloration of the dye. An attempt was accordingly made to form the dianilino-*o*-benzoquinone from catechol (as evidence of oxidation to the quinone) in the presence of methylene blue, with air excluded from the reaction by a deep layer of paraffin oil.

The test mixture contained 2 ml. of a thin suspension of human type bacilli (H 37) ground in physiological saline, 3 ml. of 1 : 5000 methylene blue solution, 5 ml. of phosphate buffer at *p*H 6·5, 0·05 g. catechol, 1 drop of aniline; after thorough mixing the liquid was covered with 2 in. of sterile paraffin oil. A control with 2 ml. distilled water in place of the bacillary suspension, and a control containing only buffer, methylene blue and aniline, properly diluted, were also prepared and covered with oil.

No reduction of methylene blue occurred during the 7 days the tubes were under observation. After 3 days there was evidently some leakage of air through the oil layer, for in the tube containing bacilli a few red crystals formed very slowly at the oil-water interface. At *p*H 6·5 and 23° methylene blue did not serve as hydrogen acceptor in this reaction.

Thermostability of the enzyme

The supernatant fluid from a centrifuged culture of human type bacilli (H 37), grown on 5% glycerol broth, was used for the tests in place of a Berkefeld filtrate, in order to avoid dilution from a steam-sterilized candle and any possible physico-chemical effects of the material making up the candle. Sterile graduated centrifuge tubes, selected for uniform shape and size of tip, were used to heat the fluid, of which 2 ml. were used for each test. The tube was clamped over a water bath so that the fluid was well immersed, heated for ½ hr. at the selected temperature, and then stored immediately in a mixture of ice and water. An unheated tube was stored in the ice as control. The temperatures used were 45, 55, 65, 75, 85 and 98°.

In this experiment the formation of dianilino-quinone was used as a test for any enzymic activity remaining. To each of the chilled tubes 2 ml. of phosphate buffer at *p*H 6·6, containing 1% catechol and 2% aniline, were added, and a chemical control tube with no culture fluid was also made up. The tubes were allowed to stand with sterile cotton plugs to admit air. The only observable difference 4 hr. after mixing was the slightly darker colour of the heated tubes compared with the unheated control; 17 hr. after mixing there were a few amorphous reddish bits to be seen in the culture fluids, but none in the control. In another 24 hr. there were clumps of definite crystals of red dianilino-*o*-benzoquinone in all the culture fluids, while there was only the merest suspicion of red material in the chemical control. There was no differentiation between tubes heated to different temperatures, or between heated and unheated controls, at this stage. (Although this was not a potent fluid compared with most of those used in the foregoing experiments, it still caused the appearance of an effect in 17 hr. which required 41 hr. to manifest itself unaided.) After another 48 hr. (89 all told) there were still very few crystals in the chemical control, but a moderately heavy red precipitate in all the culture fluids. The tubes were centrifuged to pack the sediment evenly in the graduated tips. There was still no obvious difference to be seen between the test temperatures, or between heated and unheated fluids. It was concluded that the enzyme is thermostable, contrary to expectations. Various plant polyphenolases have been reported as thermostable, according to Haldane [1930].

Separation from culture fluid of a protein possessing enzymic activity

The amount of red dianilino-quinone formed, again served as a rough measure of the enzymic activity, using catechol as substrate. The fluid used was that decanted after centrifuging a 5% glycerol broth culture of human type bacilli

(H 37). Freshly prepared 50% trichloroacetic acid was used as protein precipitant, in the proportion of 1 vol. acid to 4 vol. fluid, as used by Seibert [1934] at a certain stage in the preparation of the tuberculo-protein known as P.P.D. The protein in four 5 ml. portions of fluid was thrown down and allowed to settle overnight. After being centrifuged the tubes were allowed to stand another 24 hr.; a light secondary coagulum formed, which was adherent to the walls of the tubes and was not affected by centrifuging. From two of the tubes the supernatant fluid was decanted, and the precipitated protein stirred up well with 5 ml. of freshly prepared 10% trichloroacetic acid, centrifuged, the wash-liquid discarded and the washing with 10% acid repeated. Then the protein was dissolved carefully in the least possible amount of $N/10$ NaOH, 1·5 ml. being required for this lot, and made up to 5 ml. with distilled water. The originally decanted supernatant fluids were carefully neutralized with 10% NaOH (to dilute them as little as possible, instead of using $N/10$ NaOH). The other two tubes, still containing protein and original reaction mixture, were likewise neutralized approximately with the strong alkali, and finally with the weak; these were to serve as controls of the possibly destructive effect of trichloroacetic acid upon the total enzyme content of the fluid. A control of neutralized trichloroacetic acid itself was also prepared. To each of these (redissolved protein, neutralized supernatant fluid, neutralized whole reaction mixture and acid control) and also to portions of the original culture fluid, an equal volume of phosphate buffer at *p*H 6·6 was added. The buffer had 1% of catechol and 2% of aniline already dissolved in it. Sterile cotton plugs permitted absorption of oxygen as usual.

After 21 hr. it was apparent from the relative volumes of precipitated dianilino-quinone that part of the enzymic activity had been carried down with the protein precipitate, and part had remained in the supernatant fluid. After another 24 hr. the dark red crystals were packed down by centrifuging and the amounts in the tubes compared. The precipitated, twice washed, redissolved protein contained about three-fifths of the original activity, while two-fifths remained unprecipitated and still active in the supernatant reaction mixture. The enzymic activity had apparently not been damaged by trichloroacetic acid, since the neutralized whole reaction mixture had as much dianilino-quinone formation as the untreated original culture fluid. As usual, the few crystals formed in the autoxidation control were negligible in amount.

Summary

Various experiments have been described wherein the human type of tubercle bacillus or a product of its growth was found to catalyse the oxidation of various phenolic substances. The properties of the active agent concerned in this catalysis, as observed under the conditions employed, suggest the formation of a diffusible, thermostable enzyme of the polyphenol oxidase type, whose behaviour is more consistent with catechol- than with phenol-oxidase, and which is not a tyrosinase. Part of the activity could be removed from a culture fluid of human type tubercle bacillus by use of a protein precipitant, and recovered from a solution of the washed protein. Methylene blue did not serve as a hydrogen acceptor for the oxidation of catechol catalysed by this enzyme.

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LVI. THE METABOLISM OF LACTIC AND PYRUVIC ACIDS IN NORMAL AND TUMOUR TISSUES

VI. OX RETINA AND CHICK EMBRYO

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In rat and rabbit kidney cortex slices, lactate is oxidized to pyruvate and the greater part of the pyruvate is removed by a cyclic series of reactions whereby 2 mol. of pyruvate are converted successively into succinate, fumarate, malate and oxaloacetate; the oxaloacetate then decomposes spontaneously to yield pyruvate in half the original amount; in tumour, brain, testis and liver, however, this cycle does not occur to any extent [Elliott & Schroeder, 1934; Elliott *et al.* 1935; 1937].

The present investigation deals with the metabolism of lactate and pyruvate by ox retina and chick embryo. The same manometric and analytical methods were used as for previous studies of this series [Elliott & Schroeder, 1934; Elliott *et al.* 1935]. Some possible intermediates, not tested with other tissues, were also used as substrates in an attempt to elucidate the mechanism of the breakdown of lactate and pyruvate.

Experimental

The concentrations of substrates in the medium were: *dl*-lactate $M/25$; pyruvate, acetate, α-ketoglutarate, β-hydroxybutyrate, formate $M/50$; fumarate, *l*-malate, oxaloacetate, citrate, oxalate $M/100$; succinate $M/100$ and $M/25$. The experimental period, except where otherwise stated, was 90 min. The terms defined in previous communications of this series were adopted for expressing results.

In these experiments manometers with a wider bore and Clerici fluid containing only thallium malonate and thallium formate were used as suggested by Dixon [1937]. These changes decreased the errors of the apparatus, so no corrections of the observed Q_{CO_2} and R.Q. values have been made.

The chemical analyses for lactate, pyruvate and oxaloacetate were carried out as described by Elliott *et al.* [1935]. Elliott *et al.* [1937] concluded from work with liver that the method previously used [1935] for the estimation of oxaloacetate as keto-body was unsatisfactory in the presence of tissue materials. However, the unexpectedly low results obtained by them were probably correct and were due to the rapid reduction by the tissue of part of the oxaloacetate to malate [see Stare, 1936; Elliott & Elliott, 1939]. The earlier method for the estimation of oxaloacetate was therefore employed.

Only typical results, representative of a large number of experiments, are given in the tables.

Ox retina

Ox eyes were obtained from the abattoir on the morning of slaughter, but it was impossible to determine the exact time of killing. Some variations in results, therefore, were possibly due to the fact that the tissue was not as fresh as desired for some experiments.

For 4 manometers the retinas of 4 eyes were used, the eyes of each pair being considered identical for purposes of sampling. The cornea, iris, lens and vitreous humour were removed and the retina, while still in place in the resulting capsule, was divided radially from the optic nerve with a scalpel and taken out in sections. The tissue was kept in Krebs's medium containing glucose through which O_2/CO_2 was bubbled. After rinsing in Ringer-Locke solution (this was done twice for experiments in the absence of glucose), the material for each vessel was drained on perforated porcelain discs over filter paper and weighed. The nasal side of the retina (C in Table I) usually had a somewhat lower respiration and glycolysis than the temporal side (A and B), so for the experimental vessels roughly equivalent weights of retina from the temporal side of each pair of eyes were used. The nasal side was used in the left-hand vessels. In some preliminary experiments, the sections of retina from the temporal side were divided parallel to the circumference (the sections nearer the optic nerve were designated as A and those nearer the circumference as B); rather wide differences in Q_{O_2} were found though the differences were not consistent. When similar radial samples of the whole temporal side were taken, variations in metabolism were smaller.

Table I. *Ox retina metabolism. Comparison of different sections*

Glucose in medium, no other substrate added

	A	B	C	A	B	C	A	C	A	B
$-Q_{O_2}$	10·6 (20·8)	12·1 (22·3)	10·7 (20·0)	10·6 (21·2)	13·4 (23·9)	8·8 (19·5)	8·1 (20·6)	6·7 (16·6)	10·0 (22·6)	8·8 (17·8)
R.Q.	0·83	0·88	0·86	0·76	0·79	0·93	0·95	0·93	0·89	0·92
Q_A	11·6	10·5	10·8	11·9	11·4	6·8	7·7	7·0	7·4	7·9

Figures in parenthesis are calculated on the basis of final dry wts.

In all cases the results were calculated on the initial dry weight, i.e. on the dry weight deduced from the wet weight taken and the wet weight/dry weight ratio of a separate sample. In some experiments values for Q_{O_2} were also calculated on the basis of the final dry weight, but these were not reliable since the tissue disintegrated to such an extent, especially after the acid was added, that it could not be removed completely from the medium.

Like brain, retina exhibits in the presence of glucose a very high initial glycolysis which falls off rapidly. This is shown by the slope of the composite curve for the experiment [see Elliott *et al.* 1937, p. 1004] and by the considerable amount of lactate formed in the left-hand vessel during the 20 min. of gassing and equilibration. The Q_{LA} of ox retina, calculated on the initial dry weights, varies between +22 and +29 for the first 20 min., between +8·5 and +9·9 for 1 hr. experiments, and between +5·1 and +7·6 for the usual experimental time of $1\frac{1}{2}$ hr.

Lactate. Lactate is metabolized by retina at a rapid rate. Part is oxidized completely, as indicated by the increase in Q_{O_2}, which corresponds roughly to the acid disappearing (1 lactate requires $3O_2$ for complete oxidation), and part is changed to pyruvate as shown by the increase in Q_{keto}. When lactate or glucose

Table II. *Ox retina*

Substrate	Glucose	$-Q_{O_2}$*	R.Q.†	Q_A	Q_{LA}‡	Q_{keto}
No addition	+	11·9 (22·6)	0·88	+7·5	+6·5	+0·5 (206 mg. wet wt.)
No addition	+	12·0 (25·0)	0·92	+5·1	+5·2	+0·1 (403 mg. wet wt.)
No addition	–	8·3 (18·7)	0·86	−0·5	−0·1	0
Lactate	–	14·5 (26·6)	0·87	−2·8	−3·8	+1·4
Pyruvate	–	16·5 (32·8)	1·24	−6·2	+2·3	−7·7
Succinate *M*/100	–	9·3 (13·5)	0·79	−1·2	+0·1	0
No addition	–	6·6	0·95	−0·4	—	—
Lactate	–	10·8	0·87	−2·3	—	—
Succinate *M*/100	–	6·9	0·80	−0·8	—	—
Succinate *M*/25	–	8·2	0·66	−0·6	—	—
No addition	+	10·7 (38·1)	0·87	+6·8	+6·4	+0·2
No addition	+	9·1 (37·2)	0·96	+6·9	+6·2	+0·5
Lactate	+	11·2 (24·7)	0·82	+7·1	+4·3	+0·2
No addition	+	11·2	0·80 (364 μl. CO_2)	+8·6 (+355 μl. CO_2)	+7·6	0
Pyruvate	+	9·6	1·34	+2·8	+5·3	−4·6
No addition	+	11·3 (22·7)	0·86	+8·3	+7·6	0
Pyruvate	+	14·3 (23·1)	1·17	+1·9	+6·9	−5·0
Acetate	+	14·1 (22·4)	0·82	+7·7	+7·3	+0·2
No addition	–	7·4 (24·8)	0·97	−0·3	0	0
Fumarate	–	8·6 (19·5)	0·89	−0·5	[+1·2]	0
Malate	–	8·0 (20·4)	0·94	−1·4	[−1·5]	+0·3
Acetate	–	8·2 (20·6)	0·94	−0·5	−0·5	0
No addition	+	11·0 (21·8)	0·94	+6·8	+5·2	0
Fumarate	+	11·2 (22·9)	0·89	+7·4	[+6·2]	0
Malate	+	11·7 (22·8)	0·87	+6·8	[+6·3]	0
Acetate	+	12·2 (23·0)	0·89	+6·5	+4·7	0
No addition	+	11·3 (20·0)	0·82	+6·4	+5·1	+0·2
Citrate	+	10·4 (18·4)	1·02	+6·1	+5·2	−0·2
No addition	–	6·8 (12·8)	1·01	−1·0	—	—
Citrate	–	7·0 (11·2)	1·03	−1·1	—	—
Formate	–	6·2 (13·3)	0·97	−0·7	—	—
No addition	–	7·5 (17·5)	0·87 (304 μl. CO_2)	+0·2 (+16 μl. CO_2)	−0·2	−0·1
Pyruvate	–	13·7 (25·6)	1·12	−3·5	+1·4	−4·9
Oxaloacetate	–	14·5 (28·0)	1·64 (1033 μl. CO_2)	−12·4 (−530 μl. CO_2)	+1·5	−7·1
Oxaloacetate, no tissue	–	—	(320 μl. CO_2)	(−360 μl. CO_2)	0	—
No addition	–	7·8 (14·3)	0·96	−1·3	—	—
β-Hydroxybutyrate	–	7·7 (13·4)	0·86	−0·2	—	—
No addition	+	10·0 (14·3)	0·85	+9·1	—	—
β-Hydroxybutyrate	+	10·3	0·94	+7·8	—	—
No addition	+	8·7	0·81	+7·5	+6·6	0
α-Ketoglutarate	+	9·2	0·93	+6·1	+5·5	+0·2
No addition	–	8·1	0·88	−0·6	—	—
Oxalate	–	8·3	0·82	−0·3	—	—
α-Ketoglutarate	–	7·5	0·90	−0·9	—	—

Anaerobic

Substrate	Glucose	Q_{CO_2}	Q_A	Q_{LA}	Q_{keto}
No addition	–	0	+1·8	+1·2	−0·1
Pyruvate	–	0·3	+1·8	−0·2?	−1·5
Pyruvate	–	0·8	+1·7	+1·6	−1·5
No addition	–	0	+2·7 (+122 μl. CO_2)	+1·2	0
Oxaloacetate	–	7·2 (319 μl. CO_2)	−5·0 (−221 μl. CO_2)	+3·9	−4·3
Oxaloacetate, no tissue	–	(276 μl. CO_2)	(−310 μl. CO_2)		

* See Table I.

† The figures for μl. CO_2, given in parenthesis under R.Q. or Q_{CO_2}, represent the total respiratory CO_2 evolution during the experimental period. The figures for μl. CO_2 under Q_A represent the total CO_2 evolved or absorbed as the result of acid changes.

‡ Figures in [brackets] under Q_{LA} refer to estimations given wholly or partly by malate.

is present a small amount of pyruvate is formed in both vessels (*c.* 0·1 mg.), the amount in the left being only slightly less than in the right, indicating a high initial rate of keto-formation and that the path of glucose oxidation is probably through pyruvate. Lactate and glucose appear to be alternative substrates for retina since, in the presence of glucose, lactate has little effect on O_2 uptake or R.Q. However, with both glucose and lactate present there is often some discrepancy between lactate and acid formation, acid formation being greater than lactate formation. This indicates the accumulation of some unknown acid which is not lactate or pyruvate. This phenomenon appears occasionally to a slight extent when glucose alone is the substrate.

In order to determine whether the tissue is damaged and respiration falls off in the absence of substrate [see Krebs, 1935], and to investigate further the unknown acid, experiments of 1 and 2 hr. duration were done. The results of these experiments are given in Table III together with values calculated by

Table III. *Ox retina: 1 and 2 hr. experiments*

Substrate	Glucose	Exp. period	$-Q_{O_2}$*	R.Q.	Q_A	Q_{LA}	Q_{keto}
No addition	–	2 hr.	6·4 (15·6)	0·94	−0·8	−0·2	0
Lactate	–	2 hr.	12·7 (27·4)	0·97	−3·5	−2·7	+0·1
No addition	–	1 hr.	9·0 (21·8)	0·91	−0·9	−0·6	0
Lactate	–	1 hr.	13·8 (24·3)	0·91	−3·0	−5·3	+0·3
No addition, 2nd hr. by diff.			3·8	1·03	−0·6	+0·3	0
Lactate, 2nd hr. by diff.			11·7	1·01	−4·0	0	−0·4
No addition	–	2 hr.	8·0	0·94	−0·7	−0·2	−0·3
Pyruvate	–	2 hr.	14·7	1·14	−4·3	+1·3	−6·4
No addition	–	1 hr.	8·1	0·85	−0·2	0	+0·9
Pyruvate	–	1 hr.	14·8	1·22	−5·1	+1·3	−6·3
Lactate	–	2 hr.	15·2	0·93	−4·0	−2·3	—
Lactate + pyrophosphate	–	2 hr.	19·8	0·97	−5·2	−4·4	—
Lactate	–	1 hr.	15·3	0·92	−3·3	−5·0	—
Lactate + pyrophosphate	–	1 hr.	22·5	0·98	−6·3	−8·5	—

* See Table I.

difference for the second hour. Without substrate, O_2 uptake falls off somewhat with time. With added lactate the Q_{O_2} is increased and falls off only slightly, with the result that during the second hour the difference between the O_2 uptakes in the presence and absence of lactate is considerably greater than during the first hour. The R.Q., with and without lactate, is usually higher for the second hour than for the first.

In the 1 and 2 hr. experiments, the discrepancies between Q_A, Q_{LA} and Q_{keto} are much more marked than in the usual 90 min. experiment. During the first hour there is a large lactate disappearance while only a part of this is accounted for by acid disappearance. Since no appreciable amount of keto-body accumulates, the formation from lactate of some as yet unknown acid intermediate is presumed. During the second hour lactate is no longer metabolized but the acid intermediate continues to be oxidized. There is quite a large acid removal, $Q_A = -4$; but no lactate removal, $Q_{LA} = 0$. A similar phenomenon appears in the work reported by Elliott *et al.* [1937] in some experiments with liver. In the presence of glucose, the formation and disappearance of the unknown acid still play a part but the effects are more variable.

Since retina is reputed to be one of the richest sources of phosphatase [Reis, 1937], it was thought that the unknown acid might be phosphoric acid.

Phosphate determinations, however, showed that this would not account for the unknown acid. Possenti [1935, 1] also found no liberation of H_3PO_4 by retina during glucose fermentation. NH_3 determinations were also carried out (by Dr J. Harand of the micro-analytical department) but these did not throw any light on the peculiar acid formation and disappearance [see also Rösch & Kamp, 1928; Stutzke, 1936; Dickens & Greville, 1933].

Another possibility was that the unknown acid which accumulated from lactate in the absence of glucose might be some intermediate such as acetate, which, although not oxidized to any extent when present as substrate, might be metabolized if a certain concentration of other metabolite, e.g. pyruvate, were also present. An experiment was done with acetate in the presence of $M/1000$ pyruvate but this showed no increased removal of acetate.

Pyrophosphate increased the respiration in the presence of glucose or lactate, but unknown acid accumulated in the first hour and disappeared in the second hour as usual (see Tables III and IV). The effects of pyrophosphate are discussed in another paper [Greig & Munro, 1939].

Pyruvate. Possenti [1935, 2] found that anaerobically retina produces little or no CO_2 from pyruvate, but aerobically the O_2 consumption is markedly increased, the R.Q. also being increased from 0·8–0·9 to above 1. Our results agree with these. The rate of pyruvate oxidation is equal to or slightly greater than that of lactate. Part of the pyruvate disappearing is reduced to lactate, the rate of lactate formation being very high at the beginning of the experiment (Q_{LA} values for the first 20 min. were +19, +20, +19·7, +22, calculated from the analysis of the medium of the left-hand vessel); part is completely oxidized as is shown by the increased O_2 uptake, raised R.Q. and acid disappearance. The presence of glucose does not seem to affect pyruvate metabolism ($-Q_{keto}$), but it is difficult to determine exactly its rate of oxidation since a part is reduced to lactate, and lactate is also formed by glycolysis. Anaerobically there is negligible decarboxylation.

One- and two-hour experiments, analogous to those with added lactate discussed above, were done using pyruvate as added substrate. In these experiments acid disappearance plus lactate formation accounted for the pyruvate removal ($-Q_{keto}$) during both periods. This is surprising since, if pyruvate be the first oxidation product of lactate, and if there be an equilibrium

Table IV. *Ox retina: arsenite and pyrophosphate*

Substrate	Glucose	$-Q_{O_2}$*	R.Q.	Q_A	Q_{LA}	Q_{keto}
Lactate	–	12·6	0·94	−3·4	−3·6	+0·8
Lactate + arsenite	–	2·1	—	+2·0	−6·7	+4·0
Pyruvate	–	12·3	1·12	−2·6	+0·9	−6·0
Pyruvate + arsenite	–	0	—	+1·3	+1·7	+1·3
No addition	–	6·4	0·93	−1·0	−0·5	−0·3
Pyrophosphate	–	7·1	0·89	−0·3	+0·2	0
Lactate	–	11·1	0·85	−2·3	−4·3	+0·5
Lactate + pyrophosphate	–	16·2	0·95	−2·9	−2·5	−0·1
No addition	–	6·9 (21·9)	0·85	−0·2	−0·2	0
Pyrophosphate	–	5·2 (12·7)	1·06	−0·6	−0·3	0
No addition	+	9·8 (26·6)	0·86	+8·4	+6·3	+0·1
Pyrophosphate	+	15·2 (27·2)	0·98	+9·9	+11·8	+0·2
No addition	–	7·4 (13·6)	0·99	−0·9	—	—
Pyrophosphate	–	9·3 (12·9)	1·00	−0·8	—	—
Lactate	–	11·1 (15·8)	0·90	−2·3	—	—
Lactate + pyrophosphate	–	14·5 (20·1)	0·97	−3·1	—	—

* See Table I.

between lactate and pyruvate, one would expect to find with added pyruvate the same apparent accumulation of acid in the first period and extra removal of acid in the second period, as was found with added lactate.

In the presence of $M/1000$ arsenite, which inhibits the oxidation of keto-acids [Szent-Györgyi, 1930; Krebs & Johnson, 1937], the oxidation of pyruvate was completely inhibited. The greater part of added lactate which disappeared was oxidized to pyruvate, but a small part was transformed into some acid not accounted for by pyruvate, perhaps the same acid which appeared in other experiments with lactate as substrate. The fact that lactate removal was greater in the presence than in the absence of arsenite might be due to the inhibition of re-reduction of pyruvate to lactate.

The discrepancy between lactate and acid disappearance contrasted with the equivalent pyruvate and acid disappearance, and the results of the experiments with arsenite, suggest that lactate can be oxidized to some acid without being first converted into pyruvate, but it is difficult to picture what this process can be.

Succinate causes only a slight increase in O_2 uptake and a lowering of the R.Q., without any appreciable acid disappearance, showing that if oxidized at all, it goes only as far as fumarate. Elliott & Greig [1937] report a very low succin-oxidase content in ox retina.

Fumarate and malate form an equilibrium mixture in the presence of retina but they do not seem to be oxidized further. In the presence of fumarate there is always an unexplained lowering of the R.Q. which does not always occur with malate. This is manifest in a lowering of Q_{CO_2} and not in an increased Q_{O_2}.

Oxaloacetate. Retina accelerates the decarboxylation of oxaloacetate slightly. Aerobically the pyruvate formed is further oxidized; under anaerobic conditions it is reduced to lactate.

Acetate does not seem to be oxidized at all by retina. It may have some unexplained effect on the metabolism since in the presence of glucose it seemed to increase the Q_{O_2} somewhat and the R.Q. was lowered, but no increased acid disappearance occurred.

Formate, oxalate and β-hydroxybutyrate do not affect the metabolism of retina.

Citrate and α-ketoglutarate, which are members of Krebs's cycle for the oxidation of carbohydrate in muscle, are not metabolized by intact retina, although Grönwall [1937] reports the presence of citric dehydrogenase in retina.

Chick embryo

Six-day chick embryos were used. Two embryos were placed in each vessel, and were snipped with scissors several times, inside the vessel, before adding the medium. Although in the aerobic experiments the O_2 uptake of the chopped and intact embryos was approximately the same, in the anaerobic experiments glycolysis was considerably higher with chopped embryos. The latter finding indicates either that damage to the tissue stimulates glycolysis, or that rates of diffusion of solutes limit the apparent rate of glycolysis in the intact embryo. With chopped embryos the variation in Q_{O_2} between duplicate experiments was small (8%). Results were calculated on the final dry weights, i.e. the dry weight of the tissue obtained at the end of the experiment.

Added glucose has little, if any, effect on O_2 uptake but it does cause an increase in acid formation. The aerobic glycolysis over the usual experimental period is small, as has already been shown by Warburg *et al.* [1924] and Needham *et al.* [1937, p. 1198], but during the first few minutes of incubation the rate of

lactic acid formation is considerable, Q_{LA} for the 20 min. equilibration in the presence of glucose being 18·2, 17·5, 17·6. In the absence of glucose, Q_{LA} for the same period is 11·6, 13·9, 12·5, 11·9, 9·0. When no substrate is present, the rate of lactic acid formation falls off rapidly and the lactate formed during the equilibration period from native substrate is metabolized giving a negative Q_{LA} for the experimental period. In the presence of glucose the rate of lactate formation, although less than the initial rate, is greater than its rate of oxidation throughout the experimental period, so that Q_A and Q_{LA} for the experimental period have positive values.

Some lactate is produced anaerobically in the absence of substrate ($Q_{LA}^{N_2}=2·5$, 4·0 for the experimental period), presumably from some intracellular precursor.

Table V. *Chick embryo*

Substrate	Glucose	$-Q_{O_2}$	R.Q.	Q_A	Q_{LA}*	Q_{keto}
No addition	+	11·5	0·99	+1·5	+2·0	+0·4†
No addition	+	10·9	1·08	+1·6	+2·3	+0·5†
No addition	–	11·9	0·95	–2·4	–1·4	–0·2
No addition	–	12·3	0·90	–2·8	–1·6	0
Pyruvate	–	11·7	1·13	–3·2	+0·6	–3·4
Lactate	–	12·8	0·93	–3·3	–4·3	+0·2
No addition	–	12·1	0·95	–2·1	–0·9	–0·2
Succinate *M*/100	–	11·5	0·93	–1·9	–0·1	–0·2
Fumarate	–	12·0	0·79	–1·3	[–0·8]	0
Malate	–	12·4	0·97	–3·1	[–0·5]	0
Acetate	–	12·0	0·91	–2·5	–2·6	0
No addition	–	12·4	0·92	–1·9	—	—
Succinate *M*/100	–	12·2	0·93	–2·5	—	—
Succinate *M*/25	–	11·8	0·91	–2·2	—	—
Oxalate	–	12·5	1·01	–2·7	—	—
No addition	+	11·2	1·06	+1·1	–1·7	+0·3
Lactate	+	10·1	0·97	+0·9	–2·5	+0·3
Pyruvate	+	11·3	1·17	–1·2	+2·9	–3·6
Fumarate	+	10·2	1·00	+0·5	[–1·2]	–0·4
No addition	–	12·5	0·83	–0·7	—	—
Citrate	–	10·7	0·78	–1·2	—	—
α-Ketoglutarate	–	10·5	0·87	–1·0	—	—
Oxalate	–	14·1	0·98	–3·0	—	—

Anaerobic

Substrate	Glucose	$Q^{+}_{+CO_2}$	Q^{+}_{+A}	Q_{LA}	Q_{keto}
No addition	+	+0·8	+ 6·5	+ 6·5	†
No addition	+	0	+10·5?	+ 6·9	†
No addition	+	0	+16·1	+13·3	—
No addition	+	+1·8	+17·3	+13·4	—
No addition	–	0	+ 2·9	+ 2·5	0
Pyruvate	–	0	+ 2·7	+ 3·8	–2·6
No addition	+	+0·6	+15·7	+13·8	0
Pyruvate	+	+0·9	+12·6	+13·4	–0·9
No addition	–	3·3? (+170 μl. CO_2)	+1·1? (+55 μl. CO_2)	+ 4·0	–0·1
Oxaloacetate	–	5·0 (+310 μl. CO_2)	– 3·3 (–204 μl. CO_2)	+ 2·1	–0·3
Oxaloacetate, no tissue	–	(276 μl. CO_2)	(–310 μl. CO_2)		

* Figures in brackets under Q_{LA} refer to estimations given wholly or partly by malate.

† Intact embryo.

‡ Figures for μl. CO_2, given in parenthesis under Q_{CO_2}, represent the total respiratory CO_2 evolution during the experimental period. The figures for μl. CO_2 under Q_A represent the total CO_2 evolved or absorbed as the result of acid changes.

The rate of anaerobic glycolysis for the equilibration period is higher, $Q_{LA}^{N_2} = +15$, $+19$, in the absence of substrate. Needham & Nowiński [1937, p. 1170], using smaller embryos, found a lower anaerobic autoglycolysis, $Q_{LA}^{N_2} = 0{\cdot}65$.

In the presence of glucose, the initial rate of anaerobic glycolysis (for the first 20 min.) gives values of $Q_{LA}^{N_2} = +32$, $+35$, but the rate falls off rapidly and much lower values are found during the usual experimental period after the time taken for equilibration.

In aerobic and anaerobic experiments, a small amount of pyruvate is usually found in the left-hand vessels after the equilibration period (*c.* 0·10 mg., corresponding to Q_{keto} $+3$, $+2$). In aerobic experiments, when no substrate is added, Q_{keto} for the experimental period is negative, showing that the pyruvate which accumulates quickly at first is then oxidized away faster than it is formed.

Lactate. As mentioned above, some lactic acid is rapidly formed during the first 20 min. equilibration period from metabolites already present in the cell, and during the experimental period part of this is oxidized. The lactate formed from native substrates seems to be almost sufficient, in most cases, to maintain lactate oxidation at its maximum rate during the experiment, since with added lactate the extra acid disappearance is usually slight and O_2 uptake is not affected.

Pyruvate. Added pyruvate has no effect on O_2 uptake, but that it is metabolized is evidenced by ketone and acid disappearance, as well as by the raised R.Q. The rate of pyruvate disappearance is about the same in the presence and absence of glucose. Some of the pyruvate is reduced to lactate. Anaerobically the only effect appears to be a reduction of some pyruvate to lactate.

Succinate, fumarate, malate and oxaloacetate are not appreciably attacked by embryo. Even fumarase activity seems to be lacking in the coarsely chopped embryo, and in this respect embryo differs from other tissues studied. Fumarate usually causes a lowering of the R.Q. as was also found with retina. Blazsó [1936] found that rat embryonic tissue did not attack fumarate or oxaloacetate. He, like Elliott & Greig [1937], found succinoxidase activity in the rat embryo, but Elliott & Greig found only slight activity in chick embryo.

Acetate, citrate and α-ketoglutarate are not metabolized.

Oxalate seems to be oxidized, as indicated by acid disappearance and increased O_2 uptake. The Ringer-Krebs medium contains Ca^{++} which precipitates with added oxalate, but a considerable amount of oxalate still remains in solution.

Discussion

Compared with other tissues studied in this series of investigations retina most nearly resembles brain, but the aerobic glycolysis is higher and the succinoxidase content is lower than in brain. Retina oxidizes added lactate and pyruvate rapidly to CO_2 and H_2O; it oxidizes succinate to some extent but does not appreciably oxidize added fumarate, acetate, α-ketoglutarate, β-hydroxybutyrate or citrate.

The oxidation of lactate seems to take place in two well-defined steps—in the first stage lactate is oxidized to some intermediate acid which accumulates; in the second stage lactate oxidation ceases and the unknown intermediate is metabolized. Further investigation of this intermediate by larger scale experiments is necessary. The fact that pyruvate oxidation does not seem to occur in two steps suggests that there may be some decided difference between the modes of metabolism of these two substrates in retina.

Embryo differs from most other tissues studied in that added glucose, lactate or pyruvate have little effect on respiration. Needham *et al.* [1937] also found

that embryo (4–6 day) has reserves of an unknown nature which permit of a long-continued aerobic respiration in the absence of substrate; but they concluded that this stored metabolite was not a lactic acid precursor since they found only a negligible amount of lactic acid formed when no substrate is present ($Q_L^{N_2}$ usually below 0·5, p. 1197). Contrary to this we find with 6-day embryos, a large initial autoglycolysis, Q_{LA} for the first 20 min. +15 and for the experimental period $Q_{LA}=2{\cdot}5$. It is possible that the enzymes are kept nearly saturated with lactate and pyruvate, and therefore addition of these substrates does not affect respiration appreciably. Added succinate, fumarate and malate have no effect at all.

Concerning the theories of Szent-Györgyi and Krebs

In ox retina and chick embryo, as in liver, brain, testis and tumours [Elliott *et al.* 1935; 1937] the metabolism of lactate and pyruvate does not follow the cycle of reactions involving intermediate formation and oxidation of succinate and malate which was found to occur in kidney cortex slices [Elliott & Schroeder 1934; Elliott *et al.* 1935]. However, the negative results found with these tissues do not prove that the catalytic systems postulated by Szent-Györgyi [1937] or Krebs [Krebs & Johnson, 1937; Krebs & Eggleston, 1938] play no part in the respiratory mechanism. It is possible to assume that the necessary enzymes occur in all the types of cells studied. For instance, malic dehydrogenase occurs in liver [Green, 1936] although added malate affects the metabolism of liver slices very little [Elliott *et al.* 1937]. But the cells of the more or less intact sliced or slightly chopped tissue may already contain sufficient of the substrates oxaloacetate-malate and fumarate-succinate to carry all the respiration according to either theory. Adding more malate might increase the formation of oxaloacetate, but the reaction would cease very early owing to the inhibitory action of very low concentrations of oxaloacetate [Banga & Szent-Györgyi, 1936; Green, 1936], unless the oxaloacetate were rapidly removed by re-reduction by donator systems (Szent-Györgyi's scheme) or by a synthesis with some other molecule to give citrate (Krebs's scheme). Therefore the rate of respiration, if determined by either of these schemes, would not be greatly affected by the addition of malate. The respiration rate would depend on the rate of reductive or synthetic removal of oxaloacetate and on the activity of the malic and succinic dehydrogenases and the various carriers. Of course the picture is likely to be altered if the tissue is disintegrated and, as a result, the malate and fumarate present are diluted. Then, as shown by Gözsy & Szent-Györgyi [1934], Stare & Baumann [1936], Krebs & Eggleston [1938] and others, addition of fumarate is necessary to keep the enzymes sufficiently saturated to maintain the respiration rate.

The case of succinate addition is rather different. The oxidation product of succinate, namely fumarate, does not inhibit succinate oxidation except in high concentration [Das, 1937]. Therefore addition of succinate should cause an increase in the respiration of intact tissue unless there is only just enough succinoxidase[1] activity to maintain the normal respiration. In all the tissues studied in this series, with the exception of embryo and certain tumours, added succinate does increase the O_2 uptake. (In fact the full increase was not observed by Elliott *et al.* [1937] with liver, brain and testis slices, owing to the rapid using up of the small amount of succinate added (3 ml. $M/100$) and possibly the entrance of diffusion effects [see also Rosenthal, 1937].) But with chick embryo and the tumours studied, the succinoxidase activity is not in excess of the

[1] The term "succinoxidase" is used as a name for the complete succinate-oxidizing system consisting of succinic dehydrogenase, cytochrome and cytochrome oxidase.

amount necessary to carry the whole respiration; actually determinations on disintegrated tissue [Elliott & Greig, 1938] have shown that chick embryo and the tumours in question are definitely deficient in succinoxidase. In chick embryo and some tumours, therefore, the normal respiration does not depend mainly upon the Krebs or the Szent-Györgyi system. In retina particularly, and in testis [Elliott *et al.* 1937, and unpublished results with large amounts of succinate], the rate of O_2 uptake is increased by adding excess succinate but not as much as it is increased by adding lactate or pyruvate. Therefore, while the respiration without added substrate may possibly go through the succinate system, the entire respiration of retina and testis in the presence of lactate or pyruvate cannot be carried by the succinoxidase system, and so neither of the two theories describes the whole respiratory system in these cases. In the other tissues studied in this series, namely kidney, liver and brain, the results provide no evidence for or against either scheme.

In the case of kidney cortex slices, actual oxidative removal of malate was found, and pyruvate removal appeared to follow a cycle of reactions involving succinate and malate as intermediate steps [Elliott & Schroeder, 1934; Elliott *et al.* 1935; Elliott & Greig, 1937]. In this case oxaloacetate formed by malate oxidation must be removed by a process which is neither reduction back to malate, nor synthesis with some molecule provided from outside the cycle, since malate, succinate etc., are removed and not regenerated. It also seems unlikely that spontaneous decarboxylation alone can account for the removal of oxaloacetate, as was first suggested. It is possible that, in kidney slices, the oxaloacetate is removed by a condensation with itself or with some other substance which is derived from it and not provided from outside the cycle. This problem is being studied. The actual transfers of hydrogen to oxygen concerned in the oxidative steps of the kidney cycle may take place via the Szent-Györgyi transportase systems.

Summary

1. The metabolism by ox retina and chick embryo of lactate, pyruvate and various other compounds has been studied. Previous papers of the series indicated that in rat kidney cortex lactate is oxidized to pyruvate and that the pyruvate is removed largely by a cycle of reactions involving the successive formation of succinate, fumarate, malate, oxaloacetate and finally pyruvate in half the original amount. In neither of the tissues dealt with in the present investigation does this cycle of reactions occur.

2. In retina, lactate and pyruvate are oxidized quite rapidly. Succinate is oxidized slightly to fumarate and there is an equilibrium between fumarate and malate but no further oxidation. Fumarate causes an unexplained lowering of the R.Q. Acetate, formate, β-hydroxybutyrate, citrate and α-ketoglutarate are not oxidized to any extent. Like liver, brain and testis, retina shows a much higher aerobic glycolysis rate during the first few minutes' equilibration than occurs for the experimental period. The intermediates in lactate and pyruvate oxidation have not been identified; manometric work shows that some acid intermediate other than pyruvate is formed from lactate during the first stage of the experiment, and later, when lactate is no longer oxidized, the unknown acid acts as substrate.

3. In embryo, added lactate and pyruvate are metabolized to some extent. Succinate, fumarate, malate, α-ketoglutarate and citrate are not attacked. The coarsely chopped tissue shows no fumarase activity. Oxalate is oxidized.

4. The results in this series of papers are discussed in relation to the theories of Szent-Györgyi and Krebs.

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LVII. THE ACTION OF LIGHT ON SUBSTANCES RELATED TO ERGOSTEROL

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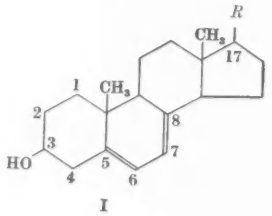
The brilliant work of Windaus and his colleagues, and of other workers, on 7-dehydrocholesterol [Windaus *et al.* 1935; Windaus *et al.* 1936; Boer *et al.* 1936; Schenck, 1937; Windaus *et al.* 1937] has culminated in the isolation of the related crystalline vitamin D_3, not only from irradiated 7-dehydrocholesterol itself, but also from the liver oil of the tunny [Brockmann, 1936; 1937; Brockmann & Busse, 1937], halibut [Brockmann, 1937], and cod [Zucker *et al.* 1938].

Of particular interest in connexion with this work is the question of the possible existence of other naturally occurring antirachitic substances, in addition to calciferol and vitamin D_3. On biological grounds Bills *et al.* [1937; 1938] consider proven the presence of such compounds in fish liver oils. Further, Windaus & Trautmann [1937] have obtained a crystalline vitamin D_4 from irradiated 22-dihydroergosterol (I *a*), while Wunderlich [1936] showed that his 7-dehydrositosterol (I *b*) possessed provitamin D activity. As Bacharach [1936] emphasized, it seemed remarkable, in view of these results, that the 7-dehydrostigmasterol (I *c*) of Linsert [1936] had no such properties.

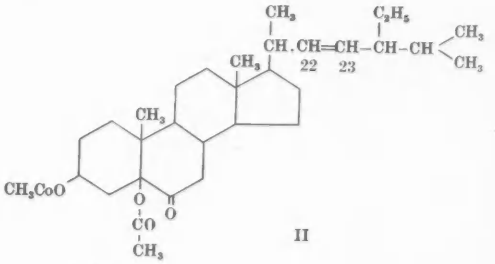
Recently, the "cholanic acid analogue of ergosterol", 3-hydroxy-$\Delta^{5,7}$-choladienic acid (I *d*) was prepared, with the use of an improved method for the introduction of the C_{7-8} double bond [Haslewood, 1938]. This method has now been applied to the preparation of 7-dehydrostigmasterol. The final product was apparently identical with that of Linsert but certain of the intermediates showed different characteristics. In the preparation, also, *3:5-diacetoxy-6-keto-Δ_{22}-sitostene* (II) was isolated.

On treatment with sunlight and eosin, in absence of air, the acid (I *d*) and 7-dehydrostigmasterol both gave crystalline insoluble products, but insufficient material was available for their complete characterization.

I (*a*) $R = -CH(CH_3).\overset{22}{C}H_2.\overset{23}{C}H_2.CH(CH_3).CH(CH_3)_2$.
I (*b*) $R = -CH(CH_3).CH_2.CH_2.CH(C_2H_5).CH(CH_3)_2$.
I (*c*) $R = -CH(CH_3).CH=CH.CH(C_2H_5).CH(CH_3)_2$.
I (*d*) $R = -CH(CH_3).CH_2.CH_2.COOH$.
I (*e*) $R = -OH$.



I



II

With the generous co-operation of Mr A. L. Bacharach, Dr E. L. Smith and Dr F. A. Robinson of the Glaxo Laboratories, the hydroxycholadienic acid and its ammonium salt, and also 7-dehydrostigmasterol have been irradiated and the products tested on rats for antirachitic activity. The tests, in the Glaxo Laboratories, and in those of the author, showed that the provitamin activity of the acid and its salt was not more than 1/125–1/400 of that of ergosterol; while irradiated 7-dehydrostigmasterol also had not more than 1/125–1/400 of the antirachitic activity of ergosterol similarly treated.

Recently, Dimroth & Paland [1939] have similarly found negative results in the case of the 3:17-dihydroxyandrostadiene (I *e*) of Butenandt *et al.* [1938]. Compounds of this group seem to show great specificity in the possession of provitamin properties.

Experimental

Analyses were microanalyses by Dr A. Schoeller. Melting points are uncorrected. Ethereal solutions were dried with anhydrous sodium sulphate.

7-Dehydrostigmasterol. Stigmasterol acetate (10 g., m.p. 142–143°) in acetic acid (500 ml.) was oxidized for a total time of 4 hr. at 50–55° with a solution of chromic anhydride (8 g. CrO_3, in 20 ml. water) and acetic acid (80 ml.); added slowly with continuous stirring during 70 min. After dilution to about 3 l. with water, the product was allowed to stand overnight and the precipitate collected and dissolved in ether. The ether was washed with NaOH and water, dried and evaporated. The residue, from ethyl alcohol, yielded white leaflets of 7-keto-stigmasterol acetate, m.p. 184–185° (Linsert: m.p. 183°). The mother liquors were diluted in three stages with water, and filtered twice from crystalline precipitates. Finally, needles were deposited, and these were collected and purified by recrystallization from methyl alcohol and methyl alcohol-light petroleum (twice). In this way, *3:5-diacetoxy-6-keto-*Δ_{22}*-sitostene* (II) was obtained as colourless needles, m.p. 189–190°. (Found: C, 74·8; H, 9·9 %. $C_{33}H_{52}O_5$ requires C, 75·0; H, 9·9 %.) 7-Ketostigmasterol acetate (1·5 g.) was reduced with aluminium *iso*proproxide, purified and the product (1·1 g.) converted into the dibenzoate exactly as described for 7-keto-3-acetoxy-Δ_5-cholenic acid [Haslewood, 1938]. The dibenzoate of 7-hydroxystigmasterol crystallized from methyl alcohol-acetone in white needles, m.p. 184–186° (Linsert: m.p. 156–158°). (Found: C, 80·9; H, 8·8 %. $C_{43}H_{56}O_4$ requires C, 81·1; H, 8·9 %.) This product (0·7 g.) was refluxed for 8 hr. with redistilled dimethylaniline (5 ml.: b.p. 191–192°). The diluted product was acidified with HCl and ether-extracted. Evaporation of the washed and dried ether and crystallization of the residue from ethyl alcohol-benzene yielded white leaflets (0·3 g.). These were refluxed for 15 min. with alcohol (50 ml.), acetone (20 ml.) and 2 *N* NaOH (2 ml.). The diluted mixture was ether-extracted, and the residue from evaporation of the washed and dried ether was crystallized from alcohol until the product (70 mg.) formed colourless needles of constant m.p. 149–151° (Linsert: m.p. 154°). 7-Dehydro-stigmasterol gave the colour reactions of ergosterol. The acetate (prepared in the usual way with acetic anhydride and pyridine at 100° for 15 min.) crystallized from alcohol-benzene in white leaflets, m.p. 169–171° (Linsert: m.p. 172°).

Effect of sunlight and eosin. (*a*) On 7-dehydrostigmasterol: this substance (15 mg.) with eosin (15 mg.) was gently boiled in a 5 ml. flask with ethyl alcohol (4 ml.) and benzene (1 ml.). While still hot, the flask was tightly stoppered, and was then allowed to stand for some weeks in a sunny place. The precipitated needles were collected, and recrystallized from benzene-alcohol. White crystals m.p. 203–204° were obtained.

(*b*) On 3-hydroxy-$\Delta^{5,7}$-choladienic acid: this acid (20 mg.), eosin (20 mg.) and ethyl alcohol (5 ml.), were treated as above in a 5 ml. flask. The product consisted of white needles, M.P. 238–239°.

Tests of provitamin activity. (*a*) In Glaxo Laboratories: 5 mg. of the compounds tested were irradiated in a quartz tube placed about 4 cm. from a mercury vapour lamp. The solutions were rapidly stirred, and a current of N_2 passed through them during the irradiation. The resulting solutions (except in the case of the ammonium salt of the acid (I), which was fed directly to rats) were evaporated, and the residues dissolved in olive oil and fed to rachitic rats. The technique of the assays was that of the "line" test. The following results were obtained:

Substance tested	Irradiation time (min.)	Activity of product
Hydroxycholadienic acid as the ammonium salt, in water (50 ml.)	2	Not more than 10 I.U. per mg.
The above acid in ethyl alcohol (25 ml.)	2, 5, 15 and 45	Not more than 10 I.U. per mg.
7-dehydrostigmasterol in ethyl alcohol (25 ml.)	3	Not more than 10 I.U. per mg.
Ergosterol in alcohol (25 ml.)	3	Not more than 4000 I.U. per mg.

(*b*) In the author's laboratory: the mercury vapour lamp was 5·5 cm. from the quartz tube. The residues from evaporation of the solutions were dissolved in olive oil and tested on Wistar rats. The standard (10 I.U.) was an olive oil solution of pure calciferol. The "line" test and X-ray technique were used in the assays. The following results were obtained:

Substance tested	Irradiation time (min.)	Activity of product
Hydroxycholadienic acid (1 mg.) in ethyl alcohol (5 ml.)	10	<25 I.U. per mg.
7-Dehydrostigmasterol (1 mg.) in ether (5 ml.)	10	<25 I.U. per mg.
0·6 mg. of the above in ether (3 ml.)	10	<25 I.U. per mg.

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LVIII. BLOOD AMMONIA[1]

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I. Introduction

Concerning the normal content of free NH_3 in human venous blood, it was previously shown [Conway, 1935] that the characteristic value of this was either zero or below the analytical level (less than 1 part in 30 millions) but that when blood was shed into an open vessel a rapid rise in the free NH_3 content occurred in the first 5 min. after shedding.[2] Observations of the same kind are described in the present paper on the rabbit (ether anaesthesia) and the fowl.

The formation and origin of NH_3 after shedding is not only of interest in relation to deamination in the blood stream itself, but also by comparison with similar processes in tissues. This blood NH_3 is here described under three headings, namely, the "alpha" NH_3 which originates immediately after shedding and for which some evidence is given that it may arise from adenosine (or possibly from minute concentrations of free adenylic acid), the "beta" NH_3 which is shown to derive after a series of stages from adenylpyrophosphoric acid, and the "gamma" NH_3 which appears to come either from vegetable adenylic acid or from adenyldeoxyribonucleotide or alternatively from substances liberating these in plasma and in the red corpusles. The "beta" and "gamma" moieties correspond to the B and A fractions of Heller & Klisiecki [1932].

From the evidence presented it would appear that most if not all the blood NH_3 forming in sterile blood collected by open shedding derives finally from adenosine.[3] This is not necessarily so for the fowl's blood which can also deaminate guanosine, cytidine and even adenine freely. Neither does it hold for mammalian blood maintained at *p*H 7·4 by suitable CO_2 tension. The "beta" NH_3 then derives very largely from adenylic acid, only part of the NH_3 appearing via adenosine.

Concerning the specificity of the deamination in blood a large number of substances containing amino (or volatile amine) groups has been studied for possible deamination in blood, but no other substances besides adenosine or associated compounds have been found to yield NH_3 (or amines) at appreciable rates or at rates comparable with the normal formation of ammonia in shed blood. Thus we have repeatedly found that arginine, if added in small amounts to shed rabbit's blood or buffered after addition at a *p*H between 7 and 8, only gives negative results. It may be noted, however, that the main point in view was to ascertain the origins of the blood NH_3 and that the conditions of the investigation did not differ very widely from the normal.

[1] A preliminary report of this work appeared in *Nature*, **139**, 1937, and **142**, 1938.

[2] Results on the normal dog similar to these findings for the human subject have been reported to us by H. Kropowski, the Pathological Laboratory, Warsaw University.

[3] The presence of adenosine deaminase in plasma was shown by one of us [Cooke, 1936] prior to Drury's communication to the Physiological Society.

II. The normal content of NH_3 in circulating blood

Methods

Experimental. The blood either from an arm vein in the resting human subject, or from the carotid in the anaesthetized rabbit (ether anaesthesia) or from the severed neck vessels in the fowl has been investigated. The method of collecting the blood has been already described [Conway, 1935] the same procedure being adopted, using a carotid cannula in the rabbit.

Revised method.

Analytical. The method of suspended absorption [Conway, 1935] with the special micro-diffusion "unit" [Conway & Byrne, 1933] has been used, but revised in certain points. The essential differences of the revised method consist first in standardizing the whole procedure with each series of determinations by means of a duplicate control on standard ammonium sulphate solutions. When this is done with the same glassware, all corrections for this and the alkali as well as temperature effects on the absorption rate are eliminated. Also it is recognized that the emission rate of NH_3 from a blood-carbonate mixture is not quite the same as from a water-carbonate mixture. The previous conclusion in this respect [Conway, 1935] was based on a few recoveries under conditions not quite ideal for the comparison, though it was pointed out that a small difference was unimportant. A very large number of such recoveries of comparatively high NH_3 quantities (to avoid appreciable fluctuations of the blood NH_3 throughout the procedure) has since been carried out.

The NH_3 formed by the alkali in addition to the preformed NH_3 in blood has also been carefully studied. It has been found to be strictly linear with the time over a few hours. No essential difference from the previous conclusions has been found, but a temperature factor has been included which, however, for the short absorption periods is scarcely significant. In Table I are incorporated the necessary details for the revised method. The allowances for the alkali action on the blood have been calculated with the help of the formula previously given [Conway, 1935] and correspond with the experimental findings.

Calculation of the blood ammonia using the revised method.

This is shown by an example.

Example 1. (All titrations carried out in duplicate, the control solution containing 2 μg./ml. NH_3-N.)

Titration of "blank" unit	...	35·6 large divisions	
Titration of "analytical" unit	...	31·3	,,
Titration of "control" unit	...	14·2	,,
Time of absorption	... ...	10 min.	
Room temperature	... ...	15°	

The amount of absorption in the analytical unit corresponds to 35·6–31·3 or to 4·3, and in the control to 21·4 large divisions (each large division of the burette [Conway, 1934] corresponds to 0·01 ml. of 0·0004–0·0005 N $Ba(OH)_2$.

$$\text{Calculation: } \frac{4{\cdot}3}{21{\cdot}4} \times 1{\cdot}15 \times 2{\cdot}00 = 0{\cdot}462\ \mu\text{g./ml. } NH_3\text{-N.}$$

The factor 1·15 derives from Table I and 2·00 represents the concentration in the control.

From the gross value of 0·462, 0·04 is subtracted to allow for the special deaminating action of the carbonate as given in Table I, column 4.

Final result = 0·42 μg./ml. NH_3-N. Before titrating a series with $Ba(OH)_2$ it is our custom to run out about 7–10 ml. from the burette, and also to have the cork of the reservoir bottle and of the soda-lime guard painted and sealed with melted paraffin. It will be seen that with the revised method changes in $Ba(OH)_2$ strength are of no consequence, but it is advisable at the same time to change the solution about once weekly. Table II summarizes a number of "recoveries" of NH_3 from blood, with the error range.

Table I

Absorption time min.	Ratio of NH_3 absorbed from water-carbonate to that from blood-carbonate mixture	The absorption as an approximate percentage of the total NH_3 in the aqueous solution %	Allowance in the final result for the special deaminating action of the alkali at different temperatures, μg./100 ml. NH_3-N				
			25°	20°	15°	10°	0°
10	1·15	52	11	7	4	3	1
12	1·14	57	14	8	5	3	1
14	1·12	61·5	16	10	6	4	2
16	1·11	65·5	19	12	7	5	2
18	1·11	69	22	14	9	5	2
20	1·11	72	25	16	10	6	2

In the above table (made out for the pyrex "unit" as made by Messrs A. Gallenkamp and Co., London) it is understood that 1 ml. blood or water is added to 1 ml. of saturated K_2CO_3 in the outer chamber from pipettes exactly calibrated for this purpose, and that the mixing is carried out with a fixed routine of 15 rotations in approximately 15 sec. The allowances in the fourth column for the special deaminating action of the alkali have a temperature coefficient of 10 % per degree.

Table II. *"Recoveries" of NH_3 by the method of suspended absorption (revised)*

Fluid examined	NH_3 added per ml. fluid μg. N	NH_3 recovered. Mean value μg. N	No. of observations	Range μg. N
Human blood	91·6	91·8	11	93·6 –88·8
Rabbit plasma	91·6	91·6	6	94·6 –87·1
Water	91·6	91·0	20	95·3 –87·1
Human blood	1·92	1·91	1	—
Human blood	0·98	0·96	5	1·05– 0·88
Human blood	0·39	0·36	2	—

The blood used in the above "recoveries" was freshly shed into an open flask. Usually 10–20 ml. samples were taken, 0·2 ml. of an NH_3 solution in saline being added and a similar amount of saline without NH_3 to a control sample.

For the first five groups 1 ml. of $N/200$ acid with indicator was added to the inner chamber and titrated after the absorption with $N/150$ $Ba(OH)_2$, and for the last three groups the customary solutions for the normal blood NH_3 were used (namely, $N/5000$ acid and $N/2000$ alkali).

Of the 37 results in the first five groups none exceed 4·5 % deviation from the mean value. The coefficient of variation for the group is 2·4 %.

For the most exact procedure possible with the method and the conditions described, the "units" after being filled and ready for the determination, are left aside on the bench for $\frac{1}{2}$ hr., the acid in the central chamber being titrated to the end-point, the fluid removed and replaced by a refill of acid and the lid replaced, after which the determination is carried out. This eliminates any small defect in cleaning and also traces of NH_3 in carbonate and fixative, and with a little experience will be found to give surprisingly good and uniform results.

The method of suspended absorption with plasma and other fluids.

The process of suspending the absorption after a short time makes the micro-diffusion method one of great flexibility in the rapid collection of numerous data, and is advisable when, generally speaking, an error range of 2–5 % for the single determination is not considered too high (the suspension being made after 10–20 min.).

With plasma, the NH_3 comes over at the same rate from a plasma-carbonate mixture as from a water-carbonate mixture so that the factors in column 2 of Table I are not used. The corrections for the extra ammonia in the last column of this table still apply and should be subtracted from the final result.

For all diluted blood and tissue extracts of the order of one in five or more, the calculation is simply the ratio of the NH_3 absorbed to that from the control multiplied by the strength of the control. The absorbed NH_3 is represented as alkali divisions on the burette.

Note on the cleaning of the "units". The following procedure has been found suitable for all purposes. After rinsing with cold and hot water, soap and a test tube brush are used, then the "units" are well rinsed and soaked in dilute acid containing indicator (about $N/200$ H_2SO_4) for 15 min. (the "units" being filled to the top). After repeated rinsing with distilled water they are dried in air.

Results

The normal resting content of NH_3 in human blood.

Using the revised method the previous conclusions [Conway, 1935] were confirmed. The NH_3 content of venous blood from the resting human subject on shedding into an open flask rises swiftly as if from zero to reach a mean value of 0·41μg./ml. NH_3-N within 5 min., after which it rises at about the rate of 0·006 μg./ml. NH_3-N/min. at 20°. The curve of the median values of successive groups of 11 observations ranged in order of time is given in Fig. 1 (upper curve). The statistical description of variability remains as before [Conway, 1935]. For the time interval of 3–10 min. after open shedding, within which the mean value alters very little, the range found in 28 observations on 12 human subjects was from 0·21 to 0·58μg./ml. NH_3-N, the range being 0·36–0·58 when observations on two subjects at the unusually low room temperature of 12° are excluded. Fig. 2 shows the course of the formation in nine subjects for whom a rather extended series was taken. The two lowest of these curves were taken at a room temperature of 12°.

NH_3 content of rabbit's blood (short ether anaesthesia).

Like the human venous blood there is a rise immediately after shedding into an open vessel. This "alpha" rise begins likewise from a concentration apparently zero—or at least not greater than 1 part in 10 million. The curve of the median values of successive groups of 11 observations on 9 rabbits is given in Fig. 1 (lower curve). (The median values are chosen in order to give the most characteristic curve.) The rabbit curve differs from the human in not reaching an apparent level after the 3rd to the 5th min. and, as will be seen in the next section, a much greater rise in the curve begins between the 10th and 20th min. Values ranging from 0·24 to 0·59μg./ml. NH_3-N were obtained from the 3rd to the 10th min. (32 observations). Fig. 3 shows graphically the variability and course of the formation for 7 rabbits within the first short period after shedding.

As shown below, the first rapid or "alpha" rise is eliminated by receiving the blood into an atmosphere containing CO_2.

The NH_3 content of fowl's blood after open shedding.

Three experiments on the fowl's blood showed a similar but much more rapid rise than for the rabbit or human subject. The blood NH_3 appears to rise here also from the zero position.

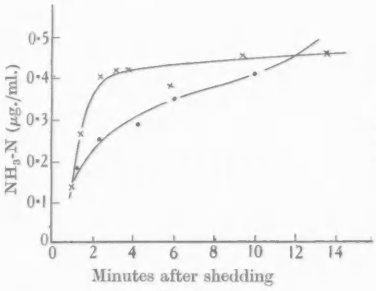

Fig. 1.

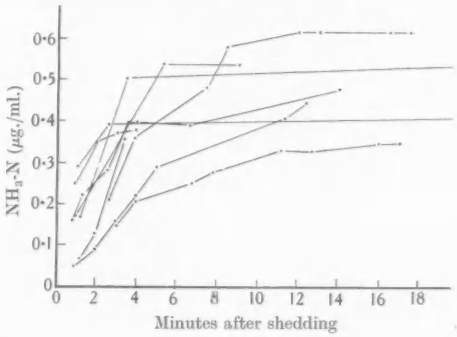

Fig. 2.

Fig. 1. Characteristic curves of the blood ammonia for man (crosses) and rabbit (dots) after shedding into an open flask. Room temperature. Each point is the median of consecutive samples of eleven observations ranged in order of time after shedding.

Fig. 2. Serial curves for nine human subjects, mostly mean of duplicates.

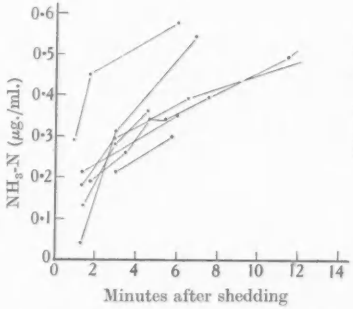

Fig. 3. Serial curves on 7 rabbits, mostly triplicate determinations.

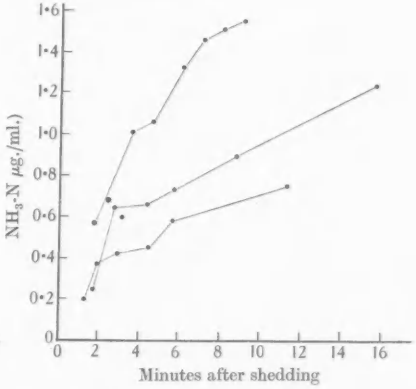

Fig. 4. Serial curves on 3 fowls, means of duplicates.

III. The NH_3 forming in shed sterile blood of man and rabbit—its stages and origins

Total NH_3. The total NH_3 forming in the blood of man and rabbit is shown in Table III. The blood over the long periods was sterilized with thymol or toluene, being stored in vaccine bottles with the usual vaccine technique of capping etc.

It will be seen from Table III that total amounts of approximately 13 μg./ml. NH_3-N develop in human blood over several days at room temperature and about 20 in rabbit blood.

Table III

Subject	Blood examined	μg./ml. NH_3-N formed after shedding					
		4 hr.	6 hr.	24 hr.	48 hr.	72 hr.	96 hr.
Rabbit	Whole blood	11·1 (3)	18·8 (3)	18·2 (7)	20·3 (3)	—	24·3 (2)
	Plasma	—	—	3·6 (5)	5·4 (2)	—	—
	Plasma (buffered with borate)	—	—	2·7 (2)	4·0 (2)	—	—
Man	Whole blood	—	—	7·4 (6)	13·1 (2)	13·8 (2)	—
	Plasma	0·7 (2)	—	1·5 (2)	—	—	2·7 (2)
	Plasma (borate buffer)	—	—	1·4 (3)	1·3 (3)	1·3 (1)	—

The numbers in brackets give the totals of individual subjects. All experiments were conducted at room temperature (mean of 18°).

The figure for the whole blood NH_3 in human and rabbit bloods after 24 hr. and longer periods agree with those of Parnas [1925] and of Stanojevic [1938] for the human subject. Earlier figures for the NH_3 in human blood after 24 hr. given by one of us [Conway, 1935] were too low owing to undue exposure of the blood, which then during this time loses NH_3 very appreciably, this exposure being carried out in connexion with a study of the effect of CO_2 loss. All vessels containing blood collected by open shedding throughout the present study were stoppered after about 20 min. initial exposure.

In human plasma 1·3–2·7 μg./ml. develop over 4 days with a mean of 1·4 μg./ml. NH_3-N. That there is practically no change from 1·4 over 3 days indicates that this may be taken as representing the total amount. The formation in rabbit plasma is greater than this. After 24 hr. about 3μg./ml. NH_3-N have developed at room temperature. The formation has not then ceased but the further increase in a subsequent 24 hr. period does not exceed 1–2 μg./ml.

The "alpha" NH_3

This formation is almost complete in the first few minutes after open shedding as shown in Fig. 1.

It amounts to a mean of about 0·4 μg./ml. N for rabbit and man. It does not appear on receiving the blood into CO_2 as already shown for the human subject [Conway, 1935] and here as in Fig. 5 for rabbit blood.

It was previously demonstrated that CO_2 in itself does not produce an apparent absence of NH_3. This has been repeatedly confirmed both for NH_3 already formed in the blood and for recoveries of minute amounts of added NH_3. When, however, the blood NH_3 is raised to the high level of 100 μg./ml. N a somewhat reduced recovery—85 %—was found with the revised method allowing the usual 10 min. absorption period. In repeated experiments no such reduced recovery was found at the 1–10 μg./ml. level, even with one atmosphere of CO_2; and even if we were to allow this 15 % it would mean a recovery of 0·34 μg./ml. N when 0·40 was really present. Such a difference is not significant for the present discussion.

The sudden lowering of the CO_2 tension in open shedding can act either by raising the *p*H of the blood or in some specific manner. We have maintained the *p*H at 7·2 by reception into a maleic acid or a phosphate buffer solution forming about one-tenth of the whole blood collected, and with results shown in Fig. 6 (curve *B*). It is obvious that this procedure if anything only increases the initial rate of the formation compared with unbuffered blood (curve *A*) so that we must conclude that CO_2 acts specifically.

It has been shown also [Conway, 1935] that carbamic acid itself is not the substance involved, but that some derivative containing an amino group is liberated, the amino group being then removed by ferment action. Adenosine could possibly fill this role, and the following is some evidence in support of such a view.

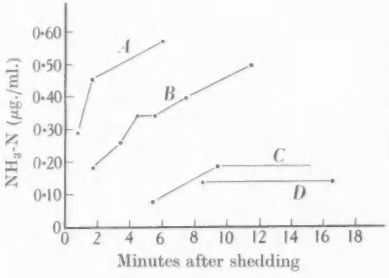

Fig. 5.

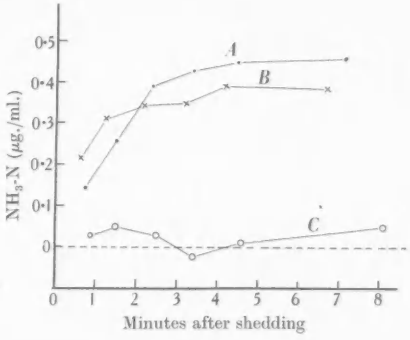

Fig. 6.

Fig. 5. Blood NH_3, 2 rabbits. *A* and *B*, open shedding; *C* and *D*, shedding into CO_2 (100 mm.).

Fig. 6. Blood NH_3, 2 human subjects. *A*, open shedding, unbuffered; *B*, open shedding, buffered (7·2 *p*H); *C*, shedding into 100 mm. tension of CO_2.

Evidence that adenosine is the amino substance involved in the "alpha" stage.

(1) Out of 51 different substances investigated of physiological importance and containing amino or volatile amine groups, only adenosine, or substances that can liberate adenosine by dephosphorylation are deaminated in mammalian blood after shedding.

(2) From evidence given subsequently small quantities of adenosine must be continually liberated in plasma.

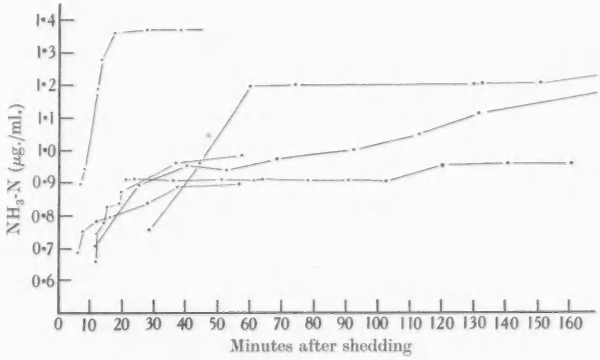

Fig. 7. Plasma NH_3 formation in 6 rabbits. Room temperature.

(3) The differences in rate of formation of the "alpha" stage in plasma and in whole blood fits in well with the adenosine hypothesis. Small quantities of

adenosine added to whole blood are deaminated in about 1 min. or less, but take upwards of 30 min. in plasma. If the "alpha" rise occurs also in plasma it cannot be faster than this adenosine deamination if adenosine is involved.

Figs. 7 and 8 show the "alpha" rises in rabbit and in human plasma, and as will be seen both take about 30 min. to reach completion.

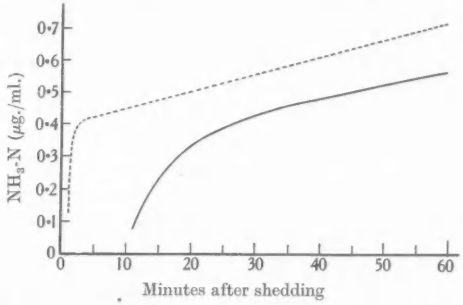

Fig. 8. Plasma NH_3 formation for 3 human subjects (mean curve) after collecting into CO_2 (100 mm. tension) with subsequent release of the gas by exposure in large flask. The dotted curve shows the NH_3 formation in human whole blood.

The rabbit plasma was obtained by centrifuging immediately after open shedding; the human by centrifuging under 100–200 mm. CO_2 tension and subsequent removal of the CO_2 by shaking a small volume in a large conical flask.

(4) The *p*H effect on the NH_3 formation in the "alpha" stage has a general correspondence with the effect on the deamination rate of adenosine. The "alpha" rise is inappreciable at 6·0 and 8·0 *p*H and seems most marked around a *p*H of 7·0. This question is again considered in the discussion at the end.

The "beta" NH_3 and its phases

The NH_3 deriving from the breakdown of adenylpyrophosphoric acid is here termed the "beta" NH_3. The NH_3 forming in whole blood after the "alpha" stage is a mixture of this NH_3 and that deriving from another source which is present alone in plasma. This latter is termed the "gamma" NH_3 and is subsequently considered.

The relation of the A.T.P. content to the NH_3 formation

Methods.

(*a*) *Ba salt determination.* The A.T.P. content was determined by a modification of the method of Parnas & Lutwak-Mann [1935] specially adapted for the absorption unit as used here. In this method the Ba salt is precipitated from a trichloroacetic filtrate and subsequently determined by the action of a muscle extract. This method allows also of a simultaneous determination of adenylic acid in the filtrate after the Ba precipitation but no appreciable quantity was found.

(*b*) *Determination of the total adenylpyrophosphate, adenyl diphosphate and muscle adenylic acid.* This is carried out simply by the action of a muscle extract on the laked blood. At first strong extracts were used (1 in 3 to 1 in 5) and the NH_3 largely removed after the addition of sodium carbonate etc. Much weaker extracts, however, may be used directly as was subsequently found (about 1 in

50 of frog or rabbit's skeletal muscle). The extract and laked blood were both buffered with maleic acid buffer (*p*H of 7·0, *M*/20 in mixture) and mixed in equal volumes for the extract action, suitable controls being set up with extract and blood diluted with similar volumes of water. The total adenyl NH_3 is formed very quickly after mixing and is complete after about 15 min. at room temperature. The formation was in all cases studied from the time concentration curves.

Results and blood NH_3 comparisons.

The determination of adenylpyrophosphoric acid in whole blood by the Ba salt method gave for 3 rabbits a mean of 9·0 μg./ml. N and 9·5 with the direct action of the muscle extract. With the Ba salt method no appreciable amount of free adenylic acid was found, and this is in obvious agreement with the results of the second method, which therefore constitutes *an easy and accurate procedure for determining the A.T.P. in blood, and particularly suitable for numerous determinations.*

In 4 hr. at room temperature the total adenyl (muscle) complex has just disappeared as such from rabbit blood and NH_3 has formed in a practically equivalent relation as shown in Table IV. With human blood it takes about 24 hr.

Table IV

Subject	Total A.T.P. plus adenylic acid, μg./ml. amino N	Change in concentration of adenyl complex μg./ml. amino-N	Change in NH_3 μg./ml. N	Time after shedding min.
Rabbit	8·4	−5·4	+7·3	180
	9·7	−9·7	+9·1	240
	10·3	−9·8	+10·3	240
Man	8·8	−5·7	+6·2	1440
	10·1	−5·7	+6·1	1440
	A.T.P. (from barium salt)	Change in A.T.P.		
Rabbit	7·8	—	—	—
	11·2	—	—	—
	8·1	−7·1	+(8·8)	225

The bracketed number in last line gives the mean NH_3-N formation in the 225 min. for many rabbits.

for a 60 % disappearance of the A.T.P. and in this time there is an approximately quantitative appearance of free NH_3—a slight excess of NH_3 being evident.

A study of the NH_3 formation in the first 4 hr. in rabbit blood is therefore in effect a study of the "beta" NH_3 over the complete range of its formation.

The figures for the total A.T.P. etc. in the two samples of human blood correspond but are somewhat lower than the data assembled by Buell [1935] for the total adenine nucleotide in human blood, passing through the uranium salt, hydrolysis of the nucleotide, removal of the uranium and determination of the free adenine nephelometrically after precipitating the silver salt. She obtained a range of 9·6–14·8 in numerous observations whereas the two samples here gave 8·8 and 10·1. Her determinations would have included both the adenosine-3-phosphoric and the adenosine-5-phosphoric acid groupings, whereas here only the adenosine-5-phosphoric acid grouping (muscle adenylic acid) would be determined.

Stages in the breakdown of adenylpyrophosphoric acid leading to the formation of NH_3

(*a*) *Carbon dioxide is allowed to escape initially.* It has been recently shown by Kiessling & Meyerhof [1938] that in muscle A.T.P. is not present as such but rather as a dinucleotide yielding A.T.P. and behaving similarly to this in all the reactions hitherto observed. However this may be for the red corpuscle, we shall consider A.T.P. here as the starting point. Seeing that adenyldiphosphate is now considered to be a stage in the breakdown of A.T.P. in muscle [Lohmann, 1935] and that no free pyrophosphoric acid is formed, we may suppose that the A.T.P. is broken down in an analogous fashion in red corpuscles and that the diphosphate is a stage leading to adenylic acid. That this is not the final stage before deamination in shed blood after escape of CO_2 is shown by the following evidence. The deaminase of adenylic acid is known to act very specifically [Schmidt, 1928] and does not act for example on yeast or vegetable adenylic acid. If we compare the actions of laked and diluted blood containing added bicarbonate ($N/10$ in mixture) on solutions of the two nucleotides made up at a *p*H of 7·0 we obtain the results shown in Table V. When the nucleotide concentration is very high—1 %—the

Table V

Added nucleotide in the laked blood mixture %	*p*H	NH_3 formation μg. N/ml./min. (multiplied by the blood dilution) Muscle adenylic acid	Yeast adenylic acid	Ratio
Rabbit A. Bicarbonate-nucleotide buffering. Blood dilution, 1 in 8·8; 0·05 *N* bicarbonate in the mixture; neutral nucleotide mixture added to laked blood				
1·0	7·5	12·2	0·890	0·07
0·5	7·7	5·45	0·238	0·04
0·2	7·9	1·32	0·132	0·10
0·1	8·0	0·037	0·032	0·85
0·05	8·2	0·0132	0·0123	0·93
Rabbit B. CO_2-bicarbonate-nucleotide buffering. Blood dilution 1 in 5. 0·025 *N* bicarbonate in the mixture and CO_2 tension 38 mm.				
1·0	7·4	4·25	0·48	0·11
0·05	7·4	0·43	0·044	0·10

deamination of the muscle adenylic acid goes very rapidly and yeast adenylic acid yields NH_3 at about 12 % of this rate. As the concentration is lowered and the *p*H shifts more closely to 8·3 the difference between the deamination rate for the two nucleotides becomes less until at a substrate concentration of 0·05 % nucleotide the NH_3 is formed from both at the same rate. This can be satisfactorily interpreted only by supposing that with low concentrations and a *p*H of approximately 8·3 the pure adenylic deaminase is no longer involved, but that the two nucleotides are being dephosphorylated to adenosine, the latter then being deaminated to inosine. (The red corpuscle is rich in adenosine deaminase as shown in a subsequent section.)

Such a conclusion is further supported by the fact that in plasma at a *p*H of 8·3 (bicarbonate buffering) the two nucleotides were found to be deaminated at the same rate independently of their concentrations (under other conditions of buffering and reaction the plasma was found to behave very differently).

We may therefore represent the stages leading to the NH_3 formation in shed blood (with escape of CO_2) as follows:

Adenylpyrophosphoric acid → adenyldiphosphoric acid + 1 phosphoric acid → adenylic acid + 2 phosphoric acid → adenosine + 3 phosphoric acid → inosine + NH_3 + 3 phosphoric acid.

(*b*) *Blood reaction maintained at* p*H 7·4 by* CO_2. The laked blood was maintained at a *p*H of 7·4 by addition of bicarbonate (approximately *N*/40 in mixture) and a tension of 35 mm. CO_2. Each tonometer used (about 300 ml. volume) contained 4 ml. mixture, the blood dilution therein being 1 in 5. A comparison of the rates of deamination of vegetable and muscle adenylic acids was made, and at all concentrations the vegetable adenylic was found to be deaminated at only a fraction of the rate (10–12 %) of the muscle adenylic acid (as shown in Table V). Clearly the main path of the deamination is here through adenylic acid and not through adenosine.

The "beta" phases. When the course of formation of the "beta" NH_3 is followed in rabbit blood at room temperature it occurs in a series of phases as shown in Fig. 9 (curve *A*). The same is observed in human blood when maintained at 38° (curve *C*) and also at room temperature in the fowl's blood (curve *B*).

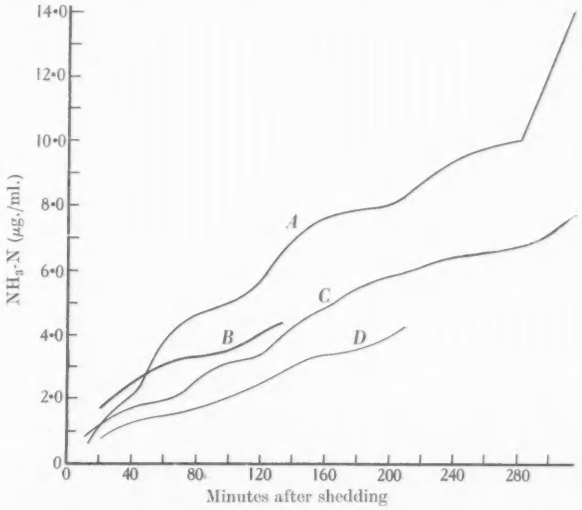

Fig. 9. Curves of ammonia formation in shed blood. *A*, rabbit blood at 18°; *B*, fowl's blood at 18°; *C*, human blood at 38°; *D*, rabbit laked blood (dilution 1 in 2 at 18°).

The curves in Fig. 9 are given as representative and not averaged from the data since this averaging tends to smooth out the inflexions owing to the appearance of these at somewhat different times from one animal to another. For the demonstration it is necessary to make about 30–40 consecutive determinations on the one blood specimen.

The NH_3 formed in each phase is of the order of 2·0–3·0 μg./ml. The reality of these "beta" phases (β_1, β_2, etc.) is very demonstrable from the fact that, at low room temperatures, the rise of the second phase may be interrupted for as long as 2 hr., the blood NH_3 remaining constant (as in Fig. 11, curve *C*). In all

the rabbit blood examined the β_1 phase was specially marked, and so far as we can judge is always easily demonstrable in a consecutive series. It begins in 10–20 min. after shedding and ends about the 50th min.

Origin of the "beta" phases. Possibilities such as sedimentation of corpuscles, phasic corpuscular permeability, the effect of sudden changes of reaction from

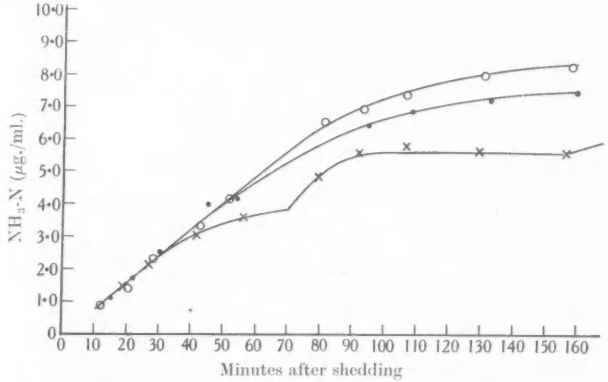

Fig. 10. The effects of fluoride and iodoacetate on the NH_3 formation in rabbit's blood. Room temperature. Upper curve (circles), iodoacetate (1 vol. *M*/5 to 20 vol. blood); middle curve (dots), fluoride (1 vol. *M*/5 to 40 vol. blood); lower curve (crosses), control blood (1 vol. of *M*/5 saline to 40 vol. blood).

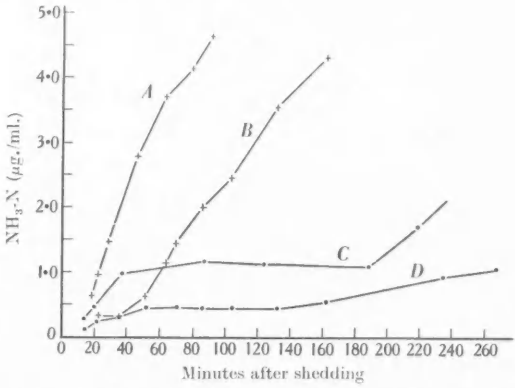

Fig. 11. Effect of adenylpyrophosphate addition to laked rabbit blood (total blood dilution, 1 in 2·45) *p*H of mixture, 8·0. *A*, adenylpyrophosphate addition (maximum NH_3-N 69 μg./ml.; *B*, adenylpyrophosphate addition (maximum NH_3-N 34·5 μg./ml.); *C* and *D*, blood controls for *A* and *B*. Blood laked and A.T.P. added immediately after shedding. Room temperature 12–13°.

unknown causes were eliminated; the blood being always shaken before an analytical sample was removed, and since the phases occur also in laked blood the question of permeability does not enter. That small changes in reaction are in themselves an immediate cause was excluded by treating the blood with alkaline phosphate (*M*/10 concentration in mixture).

From a consideration of the stages of the breakdown of A.T.P. given above, it might be thought at first that these were related to the phasic deamination. What appears to be immediately involved however is the re-esterification of the formed adenylic acid and possibly adenyldiphosphate, because the action of iodoacetic acid or fluoride converts the "beta" formation into a smooth curve. This is shown in Fig. 10. Small amounts of the reagents were added to the blood so as to make a total $M/200$ solution. The effect is a greater rapidity of NH_3 formation after $\frac{1}{2}$ hr. and a smooth curve. If such reagents are increased to $M/50$, or over, the opposed effect of a diminished rate is obtained. This we may explain by an inhibition of the initial dephosphorylation of the adenylpyrophosphate.

Adenylpyrophosphate addition and the "beta" NH_3

Neutral sodium adenylpyrophosphate, prepared by Lohmann's method [1931] passing through the Ba salt, was added to laked blood producing an increase in the amino-N of 34·5 μg./ml. N, the blood being 2·45 times diluted. In a second experiment adenylpyrophosphate was added in twice the previous amount. The course of the NH_3 formation was investigated over 4 hr. and the results summarized in Fig. 11. It will be seen that in the first experiment (curves *B* and *D*) the adenylpyrophosphate produced no increase in the NH_3 formation until about 50 min. had elapsed, when the curve rose abruptly. Twice the amount of added substance showed an earlier increase (curves *A* and *C*) and the curve of formation had a slope twice as steep.

With the laked blood without any addition (but diluted to the same extent) the β_2 phase was very long delayed, but then appeared with a well-marked rise. The slow appearance of this phase could be attributed to the unusually low laboratory temperature of 12–13°.

In the blood therefore and also probably formed after the dephosphorylation in the breakdown of the A.T.P. there is a substance which either inhibits this breakdown or brings about a re-esterification.

As is well known from re-esterification in muscle extracts [Parnas *et al.* 1935], phosphopyruvic acid can bring this about and the prevention of the formation of phosphopyruvic acid by fluoride or iodoacetic acid will lead to a more rapid disappearance of the A.T.P. and increase in the NH_3 formation. This is what we have seen to be the case on adding iodoacetic acid and fluoride ($M/200$) to blood. It would be easier, however, to explain the phasic or cyclical NH_3 formation if a substance actually inhibiting the breakdown of A.T.P. instead of re-esterifying were found. If we were to suppose such a substance effective above a certain concentration and that it was at the same time being continually removed, a brief consideration would show that it could bring about the "beta" phases under discussion.

Table VI

Blood mixture	Total A.T.P. present as μg./ml. amino-N in mixture	μg /ml. NH_3-N formed in 240 min.	1200 min.
I	69 + 4 = 73	16·2	32·0
II	34·5 + 4 = 38·5	6·4	15·8
Controls for I and II	None added + 4 = 4	1·3	3·1

Fresh rabbit blood was laked and neutral adenylpyrophosphate added to I and II samples, similar volumes of 0·9 % saline being added to control samples. The blood dilution in the mixture was 1 in 2·45.

From the above experiments it appears also that A.T.P. increases the deamination rate in blood in proportion to its concentration in laked blood. Table VI

summarizes the data in this respect. The NH_3 formation for the lowest concentration of A.T.P. in Table VI (blood to which no A.T.P. was added) is somewhat higher than might be expected from the linear relation, but under the conditions this will no doubt include in comparatively appreciable amount the "gamma" NH_3 subsequently discussed.

The pH effect on the "beta" NH_3 formation

This was examined for rabbit blood, *M* or *M*/2 buffers being added to produce *M*/20 concentrations in the total mixture, and the NH_3 formation examined over several hours. The buffers used were maleic acid, phosphoric acid, CO_2 on the acid side and bicarbonate and carbonate on the alkaline. The *p*H determinations were carried out either electrometrically on the plasma separated under paraffin or colorimetrically on saline equilibrated across collodion sacs containing blood.

The investigation was carried out from a *p*H of 7·0 to about 10·5. Fig. 12 summarizes numerous observations, the curves *A*, *B* and *C* representing the 3, 2 and 1 hr. formations. The alkaline optimum lies between 8·5 and 9·0, though at 8·0, 9·1 or 9·2 it is very little less. Beyond 9·5 or 10·0 the NH_3 becomes no doubt appreciably increased by the alkaline action on other sources of NH_3. This range of the optimum *p*H agrees with that for the breakdown of adenylpyrophosphate by liver extract, as shown by Barrenscheen & Lang [1932].

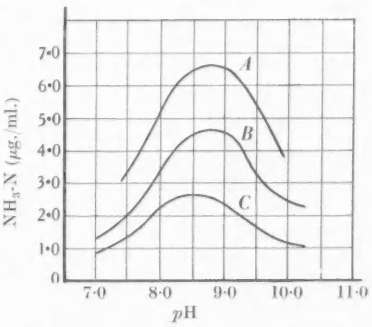

Fig. 12. The *p*H effect on rabbit's whole blood at room temperature 18°.

The *p*H effect on the blood NH_3 formation was investigated by Parnas & Heller [1924] who found that it was reduced to a very low figure at a *p*H of 9·3 and they put the optimum at about 8·1. The difference between such results and those described are due to the use of borate buffers by Parnas & Heller. These have been shown to inhibit dephosphorylation [Hommerburg, 1935] and their effect may be thus indirectly explained in this fashion. The specific quality of the borate buffers in inhibiting the nucleotide has been subsequently recognized and used, e.g. by Heller & Klisiecki [1932].

The *p*H effect as found in such experiments and summarized in Fig. 12 demonstrates at least that the optimum deamination (on the alkaline side) occurs at the same or approximately the same *p*H as the alkaline phosphatase optimum—an expected result when we consider the original source as A.T.P. As shown in a subsequent section, the type of buffer is of the greatest importance in studying pure adenylic acid deaminations.

Salt action. Saline alone will inhibit the NH_3 formation when added beyond a molar concentration of 0·1 and at 1·0 *M* the influence is very marked.

Defibrinated blood and the "beta" NH_3. Defibrinated rabbit blood shows a very similar NH_3 formation to oxalated blood. It is slightly faster at first and somewhat slower later, but is otherwise the same. The comparison was made on blood from the same rabbit (two experiments).

The "gamma" NH_3

This NH_3 derives from a precursor in plasma and corpuscles and its formation is best studied in separated plasma in which no NH_3 derives from A.T.P. or muscle adenylic acid after shedding.

The formation in plasma is very slow. After 24 hr. 1·5 μg./ml. are present in human and a little over 3 μg./ml. N in rabbit plasma. The figure for human plasma scarcely increases beyond that of the 24 hr. level, as shown when buffered with borate which acts also as a preservative. In rabbit plasma the NH_3 formation is not ended in 24 hr., but goes on at reduced rates as the time advances. The following facts are significant in deciding what the precursor of this NH_3 may be.

(1) *Specificity of deamination in plasma.* Deamination in plasma as in whole blood is extremely specific. As subsequently shown, out of 51 substances examined, of physiological significance and containing an amino or amine group, only adenosine or a few substances yielding adenosine by dephosphorylation are deaminated in mammalian blood after shedding, the CO_2 being allowed to escape.

The substances capable of yielding adenosine by dephosphorylation include the two adenylic acids, and presumably A.T.P.

There are other possible combinations which can yield adenosine, for example, the co-ferment of Warburg, which however is present only in the red corpuscles and only to about 2 % of the total adenyl complex.

It is possible also that the "gamma" NH_3 derives from the adenine deoxyriboside which in turn may be formed from the corresponding nucleotide.

(2) *Elimination of free muscle adenylic acid or of A.T.P. as possible precursors of the "gamma" NH_3.* No free muscle adenylic acid or A.T.P. exists in mammalian plasma as shown by the action of voluntary muscle extract. This gives no increased formation of NH_3, three experiments of the following type being very carefully carried out. In these the blood from a rabbit was centrifuged at once, the plasma removed and the following tubes set up.

(*a*) 2 ml. plasma + 0·5 ml. water + 0·1 ml. buffer.

(*b*) 2 ml. plasma + 0·5 ml. extract + 0·1 ml. buffer.

(*c*) 2 ml. water + 0·5 ml. extract + 0·1 ml. buffer.

(*d*) 2·5 ml. water + 0·1 ml. buffer.

The buffer was 0·2 *M* neutral phosphate (6 vol. of 0·2 *M* Na_2HPO_4 and 4 vol. 0·2 *M* KH_2PO_4) and the muscle extract a 1 in 5 extract of voluntary muscle made up 3 hr. previously from another rabbit.

The tubes were left aside for about 1 hr. when the NH_3 content was determined in each by triplicate analyses. The water used was NH_3-free and subtracting the trace of NH_3 in (*d*) from that found in the other tubes, we get from (*a*) the NH_3 in the plasma, from (*b*) the NH_3 in plasma and extract after the action of the extract, and from (*c*) the NH_3 in the extract. In exp. 1 of the group, the figures were 0·62, 5·42 and 4·72 μg. N/ml. respectively, showing 0·08 μg. N/ml. increase due to the extract, a quantity so small that it could arise as an analytical error. The two remaining experiments gave each a zero increase. In one of these the tubes were incubated for 2 hr. at 38°. Under similar conditions small amounts of added adenylic acid were deaminated in a small fraction of the time allotted for the above extract action.

(3) *Elimination of free adenosine as precursor.* Adenosine itself cannot be directly involved since it is deaminated too rapidly, small amounts disappearing in at most a few minutes in whole blood and being also deaminated with some rapidity in plasma.

(4) *Dephosphorylation and the* p*H effect.* From the foregoing evidence it would appear that likely precursors are vegetable adenylic acid, or alternatively the adenine deoxyribonucleotide. With either of these substances we may suppose a preliminary dephosphorylation with subsequent deamination of the nucleoside. At the *p*H reached by plasma after shedding with escape of CO_2 vegetable and muscle adenylic acids yield NH_3 at practically the same rate (Table VII) and this can be explained only by a preliminary dephosphorylation.

Table VII

Concentration of nucleotide in mixture	*p*H	NH_3 formation in plasma mixture over 24 hr., μg. N/ml. in: Muscle adenylic mixture	Yeast adenylic mixture	Control plasma mixture
Rabbit A. Bicarbonate buffering. Nucelotide mixture added at *p*H 8·3. 0·1 bicarbonate in mixtures. Plasma dilution 1 in 2·5. Room temperature				
0·80	8·3	8·92	8·32	1·2
0·16	8·3	9·32	7·94	
0·04	8·3	9·32	9·82	
Rabbit B. Nucleotide buffering. Neutral adenylate added to plasma in equal volumes. Plasma dilution 1 in 2·0				
1·0	7·6 (at end)	35·0	5·8	1·8
Rabbit C. Nucleotide-maleic acid buffering. Maleic acid *M*/20 in mixture. Plasma dilution 1 in 2·5				
1·0	7·0	72·6	6·3	1·4
0·1	7·0	6·3	2·3	

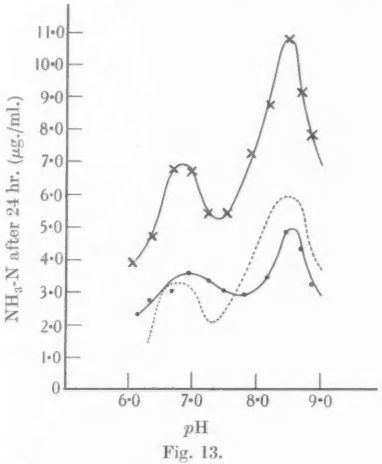

Fig. 13.

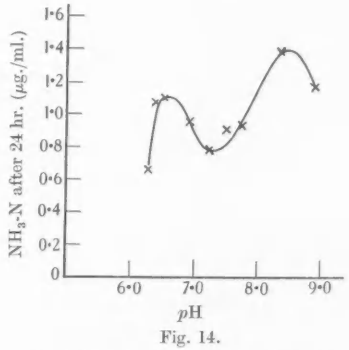

Fig. 14.

Fig. 13. The *p*H effect on the 24 hr. formation of NH_3 in rabbit plasma (lower curve) and the effect of adding vegetable adenylic acid thereto (upper curve). The dotted curve gives the extra NH_3 formed from the adenylic acid.

Fig. 14. The *p*H effect on the 24 hr. formation of NH_3 in human plasma.

Assuming the substance to be vegetable adenylic acid and the deamination to follow on a preliminary dephosphorylation, then the curve of formation of the

"gamma" NH_3 with respect to the *p*H of the buffer mixture may be expected to show two peaks, one about *p*H 8·5–9·0 and the other somewhat to the acid side of neutrality. Such double peaking has in fact been very satisfactorily demonstrated with the peaks at 6·8 and 8·6 respectively (Figs. 13 and 14). Not only did the normal plasma NH_3 appear with a double peaking when plotted against the *p*H but the extra NH_3 formed after adding vegetable adenylic acid showed an almost identical curve with peaks in the same position (dotted curve in Fig. 13). The buffer mixture used consisted of a phosphate-citrate-borate solution containing equivalent amounts of the three substances and to which small varying amounts of *N* HCl or of *N* NaOH were added. To 1 vol. plasma 0·35 vol. of the buffer mixture was added to make approximately an *M*/10 solution with respect to each buffer constituent.

After these experiments a careful examination was made of guanylic acid, cytidylic acid, guanosine and cytidine additions to plasma, for it seemed a possibility that these acids with their derived ribosides might be involved. Cytidine was prepared by the method described by Levene & Bass [1931]. The other substances (British Drug House preparations) were first purified from traces of NH_3.

Observations were carried out over 24 hr. and at a *p*H of 6·7—corresponding to the "acid" peak. Guanylic acid, guanosine and cytidine gave negative results. Cytidylic acid formed NH_3 at a similar rate to vegetable adenylic acid. This was due to the fact that commercial cytidylic acid contains vegetable adenylic acid as an impurity. A purer product made in the laboratory gave rise to no NH_3 as found in earlier experiments on whole blood.

(5) *The total organically bound acid-soluble phosphate in plasma and the increase of the free inorganic phosphate.* It is obvious that, if the "gamma" NH_3 derives from vegetable adenylic acid or the adenine deoxyribonucleotide, organically bound "acid-soluble" phosphate must exist in corresponding quantity in plasma. It is estimated (e.g. Kay [1931]) that for dog's plasma this is about 3μg. P/ml., which corresponds approximately to 1·4μg. P/ml. or an amount corresponding to the "gamma" ammonia in human plasma. We have carefully determined the acid-soluble organically bound phosphate in three rabbit plasmas, using the spectrophotometric method as described by Bomskov [1932]. Separate curves were used for the free inorganic and the total acid-soluble phosphate, constructed from standard phosphate brought through the whole procedure. The photometric readings were made by an independent observer. In these rabbit experiments (Table VIII) the mean inorganic phosphate was markedly higher

Table VIII

	Free inorganic P in plasma μg./ml.		Total acid soluble P in plasma μg./ml.	
Rabbit	Immediate	After 24 hr.	Immediate	After 24 hr.
I	69	72	75	76
II	74	77	84	84
III	73	75	78	78
IV	92	97	—	—
V, VI and VII	62	—	—	—

than that in normal human plasma, being 72·0μg. P/ml. The total acid-soluble phosphate was 79·3μg. P/ml. The organically bound P, being therefore 7·3μg./ml., would account for the first 24 hr. formation of NH_3-N in rabbit plasma (about 3μg. N/ml.).

It will appear also that if a nucleotide is being dephosphorylated before deamination that the free inorganic phosphate may be expected to show some increase over the 24 hr. period. In each of 4 exp. this increase was found, the mean value being 3·2 μg. P/ml.—corresponding to 1·5 μg./ml. Though this figure is under the expected amount if all the "gamma" NH_3 proceeds from nucleotide dephosphorylation and subsequent deamination, yet it is quite possible that inorganic phosphate may decrease slightly by ester formation.

(6) *Rate of plasma NH_3 formation and of adenylic acid deamination.* If vegetable adenylic acid is a source of the NH_3 formation in plasma, then the rate of the extra deamination on adding yeast adenylic acid should correspond with the normal formation. As an example of this correspondence the following experiment may be cited. To 2 vol. of plasma 0·7 vol. of the citrate-phosphate-borate-buffer and 0·1 vol. of water were added producing a *p*H of 8·6 (alkaline optimum). A similar mixture was set up 0·1 vol. of 2 % sodium adenylate. In the first 4 hr. the NH_3 was formed at a mean rate of 0·35 μg./ml. N/hr. in the first mixture and at 0·40 in the second. In the next 20 hr. the mean rate in the first fell to 0·14 μg./ml. N/hr., but in the nucleotide mixture remained at 0·38. The obvious explanation here is that the concentration of the nucleotide in plasma already present was somewhat above the level at which the substrate concentration ceases to have effect, but as the deamination proceeds a point is reached at which the deamination rate is affected by the concentration and the NH_3 formation falls. In the mixture with added nucleotide it continues unchanged.

(7) *Direct determination of adenine nucleotide in plasma.* If adenine nucleotide exists in plasma then the method of Buell & Perkins [1928] (recently modified by Buell [1935]) could be considered applicable to its determination, whichever modification it may be. In this method the blood nucleotide after deproteinization by trichloroacetic acid is precipitated as the uranium salt, the nucleotide being then hydrolysed to adenine by H_2SO_4 and the adenine determined nephelometrically as the Ag salt—subsequent to the removal of the uranium by NH_3. The method was applied here to plasma. To 2 vol. of plasma 3 vol. of 13·3 % trichloroacetic acid were added, and the method carried out on each plasma in triplicate, including triplicate blank determinations and duplicate recoveries of added amounts of the vegetable nucleotide (of the same order as the expected content in plasma). The recoveries so far have been rather poor and variable (up to 60 %) but the existence of the nucleotide in plasma appears to be proved, in amounts which can be only provisionally stated as about 5 to 10 mg./100 ml.

Summarizing this evidence, one may say that the "gamma" NH_3 in plasma is formed either altogether or in large part from an adenine nucleotide other than muscle adenylic acid, and that the formation occurs after a preliminary dephosphorylation. Vegetable adenylic acid fulfils the requirements so far considered for the precursor of the "gamma" NH_3 in plasma.

IV. Substances deaminated after addition to shed blood

A large number of substances containing an amino or substituted amino group was investigated for deamination on adding to shed blood. The object of this investigation was not to study the full possibilities of blood deamination, but rather to make clearer the sources of the blood NH_3. With this end in view the conditions established were never very far from those in shed blood, the *p*H range being from 6·7 to 8·3.

Apart from adenosine and high concentrations of muscle adenylic acid (both of which are rapidly deaminated by whole blood) the usual procedure adopted was to introduce into a sterilized vaccine bottle 10 vol. of laked blood (1: 1) or of plasma with 8 ml. of thymol water and 3 ml. of neutralized substrate (with or without buffer). A small crystal of thymol was generally added as well. Very occasionally toluene was used instead of thymol. The bottle was capped and sealed with the usual vaccine technique, fluid for analysis being removed through a sterilized hypodermic needle introduced through the cap. The investigations were extended as a rule to 24 hr. or longer at room temperature (mean of 18°). The procedure was occasionally varied with regard to blood volume etc. The whole blood dilution throughout was from 4·2 to 2·5 times, and for plasma from 2·1 to 1·2 times. Two control bottles were always set up, the substrate being omitted from one and the blood from the other, the same volume and other conditions being maintained. The deamination of the substrate in the blood mixture was always reckoned by comparison with these controls.

In the expression of the resulting deaminations where positive results were obtained these are given as μg./ml. N/min. with respect to the original blood.

Negative results were obtained for mammalian blood with the following substances: arginine, alanine, aspartic acid, glycine, glutamic acid, *l*-histidine, ornithine, *l*-tyrosine, valine, proline, *l*-tryptophan, *l*-cystine, cysteine, glutathione, egg-white digest with pancreatic juice (boiled), acetamide, propionamide, asparagine, histamine, adrenaline, tyramine, acetylcholine, choline, colamine, betaine, neurine, glycocyamine, glucosamine, guanidine, ethylguanidine, dimethylguanidine, creatine, creatinine, uric acid, vitamin B_1, allantoin, guanine, adenine, cytosine, guanosine, cytidylic acid, guanylic acid, methylamine, ethylamine, propylamine, butylamine and *iso*amylamine.

Positive results for mammalian blood were obtained with adenosine, adenylic acid (muscle and vegetable), adenylpyrophosphate and thymus nucleic acid (slight action) as shown in Table IX. In fowl's blood guanosine, guanine, adenine, cytidine and cytosine were in addition deaminated rather freely.

In all cases of positive action sufficient time was left for the production of at least 1μg./ml. NH_3-N and usually considerably more. This amounts to about 10 large divisions on the burette using the technique described. It will be seen that the deamination rate of adenosine is very much greater than that of adenylpyrophosphate, vegetable adenylic or muscle adenylic acid when this is at a level of 0·1 % (far exceeding any possible normal value). When the concentration of muscle adenylic acid is increased to as much as 1 % the deamination rate surpasses even that of adenosine. The significance of this is subsequently considered. The order of the adenosine deamination rate is also much greater than that of guanine, adenine etc. in fowl's blood. Guanosine is attacked much more rapidly than adenine, guanine, cytosine and cytidine in fowl's blood, and guanosine deaminase also exists in the fowl's plasma.

Concerning the sources of the substances used in the above investigations, adenylpyrophosphoric acid was prepared by Lohmann's method [1931], muscle adenylic acid by that of Ostern & Parnas [1932], later supplies being received from Messrs Henning, Berlin. Cytidylic acid, cytidine and cytosine were prepared by the methods of Levene & Jacobs [1911], Levene & Bass [1931] and Levene [1903]. Ornithine, betaine and neurine were supplied by Dr Schuchardt, Görlitz, and the remaining substances by the British Drug Houses.

Table IX

Substance	Subject	Blood used	Substrate conc. %	μg. N/ml./min. (× dilution)	pH	Buffer	Temp.
Adenosine	Man	Whole	0·2	1·25 (4)	7·0	Phos.	18
		Plasma	0·2	0·23 (1)	6·7	Maleic	38
	Rabbit	Whole	0·2	5·85 (5)	7·0	Phos.	18
		Plasma	0·3	0·040 (1)	7·0	Maleic	18
	Fowl	Whole	0·03	6·590 (2)	7·0	Phos.	21
		Plasma	0·03	0·007 (1)	7·3	Phos.	25
	Frog	Corpuscles	0·1	6·040 (6)	6·7	Maleic	16
		Plasma	0·1	0·550	6·7	Maleic	16
	Arenicola	Whole	0·03	0·058 (3)	6·7	Maleic	16
Adenylic acid (muscle)	Man	Whole	1·0	3·480 (1)	7·0	Maleic	18
			0·05	0·066 (1)	7·0	Maleic	18
	Rabbit	Whole	1·0	8·900 (2)	7·0	Maleic	18
			0·1	0·020		Maleic	18
		Plasma	1·0	0·126 (1)	7·0	Maleic	18
			0·1	0·011			
			1·0	0·014 (2)	8·3	Bicarb.	20
			0·1	0·014	8·3	Bicarb.	20
Adenylic acid (yeast)	Rabbit	Whole	1·0	0·80 (2)	7·0	Maleic	21
			0·1	—	7·0	Maleic	21
			1·0	0·748 (2)	8·3	Bicarb.	19
			0·1	0·037	8·3	Bicarb.	19
		Plasma	1·0	0·011 (1)	7·0	Maleic	18
			0·1	0·004 (1)	7·0	Maleic	18
			1·0	0·013 (2)	8·3	Bicarb.	20
			0·1	0·015	8·3	Bicarb.	20
Adenylpyrophosphoric acid	Rabbit	Whole	0·28	0·169	8·0	No extra	19
			0·15	0·065 (2)	8·0	Buffer	19
			0·015	0·013	8·0		19
Nucleic acid (thymus)	Rabbit	Whole	0·3	0·004 (2)	8·0	No extra	18
			0·3	0·010 (1)	8·0	Phos.	40
			0·3	0·002 (1)	8·0	Phos.	18
Adenine	Fowl	Whole	0·04	0·029 (1)	7·3	Phos.	21
			0·04	0·038 (1)	8·0	No extra	21
Guanine	Fowl	Whole	Sat.	0·060 (1)	7·3	Phos.	21
Cytosine	Fowl	Whole	0·2	0·088 (1)	7·3	Phos.	21
Guanosine	Fowl	Whole	Sat.	0·309 (1)	7·3	Phos.	25
		Plasma	Sat.	0·009 (1)	7·3	Phos.	25
Cytidine	Fowl	Whole	0·1	0·034 (1)	8·0	No extra	21
Adenine-cystosine dinucleotide	Fowl	Whole	0·1	0·014 (1)	7·3	Phos.	25

The bracketed data were obtained from the same blood. The bracketed numbers give the total animals. Buffer strengths ranged from 0·1 to 0·2 *M*.

Discussion

Since small amounts of NH_3 are probably liberated continually into the blood stream it may be more correct to hold that the characteristic value of the blood NH_3 is below the analytical level (about 0·002–0·003 mg./100 ml. NH_3-N) than that it is zero; though as previously noted [Conway, 1935] the extrapolation of the mean curve formation gives no free NH_3 at zero time.

Among the more reliable results obtained by other observers the nearest to this was found by Markert [1934; 1936] using a slight modification of the

Parnas-Heller method in which he determined the NH_3 spectrophotometrically. Markert placed the NH_3 content in human blood at less than 0·010 mg./100 ml. NH_3-N and in 30 experiments found no trace of free NH_3 therein

For the first or "alpha" rise after shedding—occurring in the first 5 min. and amounting to about 2 % of the total NH_3 that forms—we have given evidence to show it may derive from adenosine. For this, however, it is necessary to suppose that adenosine in minute concentrations is in some way protected by CO_2 from the action of the ferment. This so far has not been demonstrated, though not fully investigated. On the other hand, an undoubted and powerful inhibition by the CO_2-bicarbonate system has been shown for adenylic acid deaminase, so that the possibility must be considered of minute amounts of free adenylic acid causing this "alpha" rise. The difficulty of explanation here would be one of rate of action. Reckoning from the effect on adenylic acid added to laked blood, about 20 % at most of minute amounts of adenylic acid are deaminated in 5 min. in unchanged blood, and this is derived from a supposition of linearity of action from high substrates downwards. As we have seen the indication is that at the lowest levels the slope of the curve is less. A further factor, however, must also be considered, namely, that the blood immediately after shedding has a temperature not far from 37° and within the first few minutes the deaminating action is no doubt very considerably greater than at 18°. It remains possible, therefore, that the "alpha" NH_3 derives from a minute amount of free adenylic acid.

However this may be, for the "alpha" NH_3 it is certain that the "beta" NH_3 derives from the breakdown of adenylpyrophosphate. In shed blood with an escape of CO_2 dioxide the path has been shown to pass through adenosine, but the deamination is mainly direct under normal conditions. This NH_3 amounts to about 1·0 mg. NH_3-N/100 ml. both for the rabbit and the human subject, and accounts for 60–75 % of the whole formation. Attention may be drawn here again to the curious phasic appearance of the "beta" NH_3 and its apparent relation to re-esterification processes. The first of these phases is the most characterized and it is only after this phase has ended that added A.T.P. produces extra NH_3 unless added in large quantities.

The "gamma" NH_3. This is at present considered for plasma alone, though it probably also forms in the red corpuscle. Evidence has been presented that this derives from vegetable adenylic acid or possibly from the deoxyribonucleotide. Assuming the precursor to be vegetable adenylic acid it is the only one of the four adenylribose compounds, including adenosine, muscle adenylic acid and A.T.P. which can remain for any appreciable time in plasma. Adenosine disappears in the fraction of a minute (it is freely permeable through the red corpuscle) and muscle adenylic acid very probably in the fraction of a second as it passes through a voluntary muscle zone, for voluntary muscle is freely permeable to muscle adenylic acid, and presumably to adenylpyrophosphoric acid.

Consequently as a carrier of adenylribose grouping to tissues and as a comparatively durable substance with the pharmacological action of its class its significance may be considerable.

Summary

1. A revised method is described for determining the blood NH_3.

2. The immediate or "alpha" rise of NH_3 after shedding (about 0·04 mg. N/100 ml.) is dependent on the escape of carbon dioxide and not on a *p*H change. Evidence is given for its deriving from minute amounts of adenosine or adenylic acid.

3. Following the "alpha" rise in rabbit blood there is a comparatively rapid formation of NH_3 in a series of stages ending in 3–5 hr. at room temperature and deriving from A.T.P. The path is largely through adenosine in shed blood with escape of CO_2, but mainly from adenylic acid if the CO_2 tension be maintained. This NH_3 in rabbit blood amounts to 50–60 % of the total forming in sterile blood (about 2·0 mg. N/100 ml.). In human blood it amounts to 80 % of this (1·3 mg. N/100 ml.) and forms much more slowly, 50 % only appearing after 24 hr. at room temperature. The optimum *p*H of its formation is 8·7.

4. An easy method has been described for determining the A.T.P. in shed blood very suitable for serial determinations, and agreeing with the much longer method of Parnas & Lutwak Mann [1935] through the Ba salt.

5. The "beta" phases of which the most marked is the first are abolished by iodoacetic acid or by fluoride (*N*/200).

6. A.T.P. added to shed rabbit's blood develops NH_3 comparatively rapidly and in proportion to the amount added. NH_3, however, does not begin to form therefrom (unless large amounts have been added) until after the first "beta" phase.

7. The plasma or "gamma" NH_3 (probably also forming in the red corpuscles) and amounting to about 1·5 and 4 mg. N/100 ml. in rabbit and in human blood respectively, appears to derive largely from yeast or vegetable adenylic acid (or possibly from the deoxyribonucleotide).

8. Of 51 substances of physiological importance containing amino or volatile groups, only the following yielded NH_3 in blood: adenosine, adenylic acid (muscle), adenylic acid (yeast), A.T.P. and thymus nucleic acid—this last giving only minute amounts.

In avian blood adenine, guanine, guanosine, cytosine and cytidine are freely deaminated.

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LIX. THE DEAMINASES OF ADENOSINE AND ADENYLIC ACID IN BLOOD AND TISSUES

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In this paper an account is given of the activity of these enzymes as they appear in blood and tissue extracts. Whereas the deaminase of adenosine acts therein as one may expect, that of adenylic acid has certain unusual characteristics. The most striking fact is the great susceptibility of the adenylic deaminase to the inhibiting action of certain anions, though the anions of maleic and citric acids are practically without effect; besides which, there are other inhibiting substances in tissues (probably anions also) but not in voluntary muscle. The result of their action is a protection of the adenylic acid as it forms in tissues generally and a simulation of a proportionality of action to substrate concentration. From such effects also it may be occasionally concluded that adenylic acid deaminase is absent or in very small concentration when in fact very appreciable amounts are present.

From this study, voluntary muscle appears as a very exceptional tissue in so far as the mean *effective* deamination rate of adenylic acid therein would appear to be of the order of 500–1000 times greater than that in other tissues. The adenosine deaminase behaves very differently and its action is practically independent of the buffer used provided that the *p*H be maintained.

The distribution of the enzymes has been studied for 36 tissues of the rabbit with results of interest; it being shown, for example, that the appendix contained the highest concentration of adenosine deaminase in the group studied and presumably in the whole body.

As regards the course which the deamination of adenylic acid takes in tissues, it is shown that not only in voluntary muscle, but also in brain, nerve tissue, the auricle of the heart and blood maintained at normal *p*H the deamination is direct, but that in six other tissues examined and probably in the remaining tissues of the body, the deamination occurs only after a preliminary dephosphorylation.

The statement in the preliminary report in *Nature* [1938] that plasma was found to contain no pure adenylic acid deaminase is now revised, since further experiments have shown that it may contain small variable amounts, but these are of the order of 0·005 % of that present in skeletal muscle. This has appeared from the study of high nucleotide concentrations (buffered at *p*H 7·0) the enzyme being practically inoperative at low concentrations.

Properties of the deaminases in blood and tissues

Methods. Water extracts of tissues ground with pure quartz sand (Merck's) were used throughout unless otherwise stated. The efficiency of extraction with water and saline extracts etc. is commented on below. For the ammonia and other estimations the procedure described in the previous paper [Conway & Cooke, 1939] was used.

Time and enzyme activity

Here we may distinguish at least two effects, firstly that on the enzyme-substrate activity and secondly the ageing or deterioration of the enzyme.

For adenosine deaminase acting for a few hours at room temperature and provided that the substrate concentration does not fall below 5 μg./ml. amino-N, the action shows a linear relation with time. The same applies for the adenylic acid deaminase when the action is negligibly small compared with the substrate concentration.

The activity of the extracts is found to decrease but little over 24 hr. at room temperature, but the deaminase of adenylic acid rapidly deteriorates at 38–40° (unbuffered or buffered at *p*H 7·0) giving only a small fraction of its activity after a few hours. Similar observations at the higher temperatures have not been made with adenosine.

Substrate concentration

Adenosine. The enzyme in laked blood and tissue extracts acts independently of the substrate concentration beyond a certain very small concentration level. From the data in Table I this level is from 2 to 5 μg./ml. amino-N for a 1 in 5 dilution of rabbit blood. The activity is proportional to substrate concentration when this is below *ca.* 2 μg./ml. amino-N or about 0·003 % of adenosine.

Table I

Adenosine concentration in mixture at zero time	Deamination in 1 min.	% of full deamination after long period
Amino-N μg./ml.	NH_3-N μg./ml.	
0·62	0·35	56·4
1·15	0·55	47·6
2·88	0·98	34·1
5·75	1·20	20·9
14·8	1·22	8·4
29·6	1·20	4·1

Blood dilution 1 in 5. Maleic acid buffer (*M*/20) at *p*H 6·8.

Adenylic acid (muscle). The effect of the substrate concentration is here very different. With laked blood and dilutions of 1 in 5 to 1 in 50 and a *p*H of *ca.* 7·0 the typical course of the deamination with increase of substrate is shown in Figs. 1 and 2. In such experiments with either human or rabbit blood the curve shows three sections. In the first comparatively short stage the increase of deamination rate with substrate is much less than in the second, beginning at about 0·1–0·2 %. From this on to 1 % and more the course is strictly linear. At or beyond 1 % it bends to reach saturation rate of the deamination. The same features of the curve may be observed independently of the buffer used, but the actual rate of deamination will be found to vary as one buffer or another is employed. Even without any added buffer (except the adenylate) the upper parts of the curve can be followed at comparatively steady *p*H (using short observation times) and found to correspond with the above description.

As shown below, about 90 % of the deamination is here proceeding by direct deamination of the adenylic acid and not after a preliminary dephosphorylation.

At the saturation level of the adenosine deaminase (about 0·005 %) the NH_3 formation from adenosine is *ca.* 1000 times greater than from adenylic acid, but at *ca.* 100–200 times this concentration the rate approaches that of adenosine and

finally considerably exceeds it. The physiological action will be confined to the lowest concentration levels.

The effect of the substrate concentration in extracts of six different tissues is shown in Fig. 3. The dilution was 1 in 40 with maleic buffering at *p*H 7·0. The

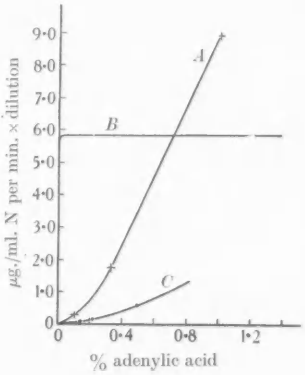

Fig. 1.

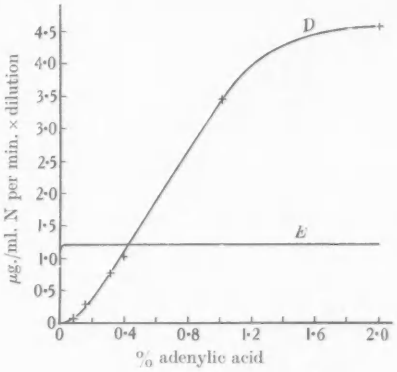

Fig. 2.

Figs. 1 and 2. Deamination rates of m. adenylic acid and of adenosine with respect to substrate in rabbit and human blood. *A*, curve of m. adenylic acid deamination in rabbit blood, with maleic acid buffer at *p*H 7·0. *B*, similar to *A* with phosphate buffer. *C*, curve of adenosine deamination with phosphate buffer, at *p*H 7·0. *D*, curve of m. adenylic acid deamination in human blood as in curve *A*. *E*, curve of adenosine deamination for human blood as in *C*.

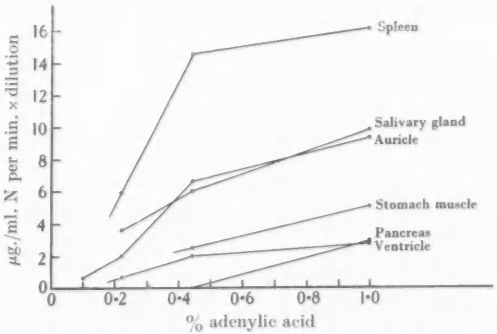

Fig. 3. Deamination of m. adenylic acid in tissue extracts. Maleic acid buffering at *p*H 7·0. Tissue dilution 1 in 50.

general effect is similar to that with blood. Voluntary muscle extract is markedly exceptional to this, the deamination rate increasing by only 10 % over the range 0·08–0·8 % and the mean value being over 70 times greater than the highest recorded in Fig. 3. Of the six tissues, however, only three deaminate the nucleotide in the main directly—these are voluntary muscle, cerebral cortex and the auricle of the heart. The others—as shown below—deaminate it almost if not quite entirely after an initial dephosphorylation, but apart from the extract of voluntary muscle the effect of substrate concentration is similar. From Fig. 3 it will appear

that at possible physiological levels—at and less than 0·1 % of the nucleotide the effective deamination rate in voluntary muscle extract is of the order of 500–1000 or more times as great as in other tissues.

Over the linear region of action of the enzyme with respect to substrate it may be pointed out that a unimolecular reaction relation will be simulated, this relation implying a proportionality of rate to substrate concentration. The underlying cause of the linearity is however quite different. (This does not contradict our previous statement of linearity of action with the time applying when the NH_3 formed is negligibly small compared with the possible total.)

The effect of dilution

With the adenosine deaminase the effect is the expected one. The total deamination rate for 1 ml. blood or 1 g. tissue is independent of the volume in which this is dissolved or dispersed. It is quite otherwise with the adenylic acid deaminase in blood and other tissues. Here the deamination per 1 ml. blood increases greatly with the dilution if the substrate concentration is not very high. This is illustrated in Fig. 4 in which dilution and deamination are expressed logarithmically.

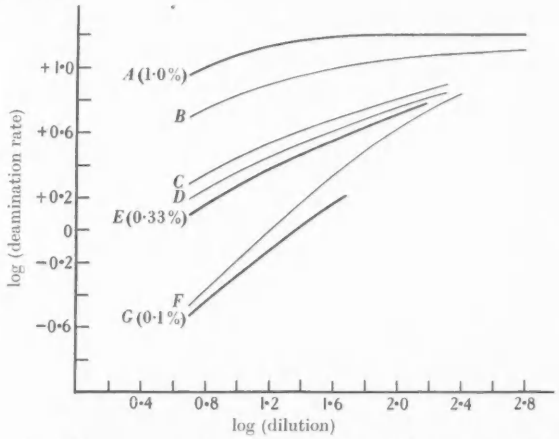

Fig. 4. Effect of dilution on m. adenylic acid deamination in blood. *A*, *E* and *G* are curves for same rabbit blood using 1, 0·33 and 0·1 % nucleotide and CO_2 and bicarbonate buffering at *p*H 7·0. *B*, 0·5 % nucleotide, self-buffering. *C*, 0·5 % nucleotide, CO_2 and bicarbonate buffering. *D*, 0·36 % nucleotide, self-buffering. *F*, 0·40 % nucleotide, maleic buffering. All at *p*H 7·0.

The ordinates in the graph give the logarithms of the deamination rates (μg./ml./min.) for 1 ml. rabbit blood independently of the volume in which it is contained. The thick lines *A*, *E* and *G* for nucleotide concentrations of 1·0, 0·33 and 0·1 % respectively are for the same rabbit blood with CO_2 dioxide and bicarbonate buffering (*ca.* *p*H 7·0). The other curves are for other blood samples and different conditions of buffering as described under the figure.

It will be seen that the general effect in which dilution causes an ultimate approach to approximately 13·8 μg./ml. NH_3-N/min., is independent of the substrate concentration. With high substrates this is quickly reached, and with low after much dilution. *Dilution at the lower substrate levels can increase the total deamination 60 times or more.*

Maleic acid buffering in blood at comparatively small dilutions appears to have an inhibiting effect on the deamination and this would appear to be associated with some precipitate formation. For tissue buffering in general with the adenylic acid deaminase it is, however, much superior to phosphate, bicarbonate/CO_2 etc. as will appear in the next section.

Specific buffer action

While the ideal procedure would here require the purest enzyme preparations, yet direct examination of very dilute voluntary muscle extracts gives the essential information. When this dilution amounts to 2000 times, the extract is still very active, and provided that the correct conditions of room temperature, buffering and *p*H are present a rate of 0·5 μg. N/min. is obtained which will produce NH_3 in somewhat over $N/500$ strength within 1 hr. In this way we examined five buffers, citric, maleic and phosphoric acids, CO_2/bicarbonate and veronal. The muscle extract dilution in the mixture was 1 in 2000, the buffer strength about $M/20$ (the bicarbonate/CO_2, $M/40$). The *p*H was carefully fixed at 7·4 and the deaminating action not allowed to proceed for longer time than required for formation of $N/5000$ NH_3. The effect on adenosine deaminase of the same buffers was studied in a similar way using laked blood (total dilution of one in eight). Table II shows the results for 0·1 and 1·0 % of the nucleotide and corresponding

Table II

		Adenylic acid deamination in 1 in 2000 voluntary muscle extract μg. N/ml./min. (× dilution)				Adenosine deamination in 1 in 8 diluted blood μg. N/ml./min. (× dilution)		
Buffer mixture at *p*H 7·4	Exp. no.	1 % nucleotide	0·1 % nucleotide	Ratio %	Exp. no.	0·7 % adenosine	0·07 % adenosine	Ratio %
Maleic	1	150	97	65	5	2·54	2·49	98
	2	696	602	86	—	—	—	—
Citric	1	352	289	82	—	—	—	—
Phosphate	1	143	3	2	6	2·71	2·56	94
CO_2/bicarbonate	3	350	3	1	6	2·64	2·82	107
	4	716	37	5	—	—	—	—
	1	42 (?)	3	—	—	—	—	—
Veronal	1	327	14	4	6	2·59	2·70	104

The strength of the buffers in the mixtures was from $M/20$ to $M/10$.

concentrations of the nucleoside. It will be seen that a change of adenylic acid concentration from 1·0 to 0·1 % affects only to a small degree the deamination with maleic and citric buffers. With maleic buffer in two experiments the reduction was to 86 and 65 % (with this we may include the effect with an extract dilution of 40, when the reduction was to 90 %). With citric buffer the reduction was to 82 %. With phosphate, bicarbonate and veronal buffers on the other hand, the deamination rate falls almost to zero or less than 3 % in the mean. Apart from its theoretical significance it is of some practical analytical importance to realize that by a suitable choice of buffer the deamination rate of adenylic acid by dilute muscle extract may be increased to over 20–30 times.

If we consider the deamination rate at 1 % nucleotide of the various buffers, citric seems the most efficient with a rate of 352 μg. N/ml./min. In exp. 1 the bicarbonate system gave the very low figure of 42, which may have arisen from

the use of 5 % CO_2/oxygen mixture from a cylinder, alveolar air being used in the other two experiments, though we cannot say why this difference should arise.

With adenosine deamination the results are very different. Here the rate is independent both of the substrate concentration (over the range studied) and of the buffer used.

The dilution was chosen so that the actual deamination rates in the blood-adenosine and the 1 %-adenylic-muscle-extract mixtures were not very different having means of 0·2 and 0·3μg. N/ml./min. respectively.

Specific inhibitors of adenylic acid deaminase in blood and tissues

The mechanism whereby a linear proportionality of deamination to substrate concentration is simulated is obvious enough from the above study. Very probably the anion of the special buffer is absorbed on to the enzyme system displacing the adenylic acid. Over a certain range a proportionality effect can be simulated on increasing the nucleotide concentration, particularly if this is not adsorbed as readily as the buffer.

The practical independence of substrate concentration seen in the muscle extracts with maleic or citric buffers and in a 1 in 40 as in a 1 in 2000 tissue dilution, does not appear in blood or general tissue extracts in dilutions up to 1 in 50. Here the bicarbonate and free phosphate concentrations are negligible (in a special blood experiment the bicarbonate was totally removed) yet the type of curve shown in Figs. 1 and 2 remains. Some other inhibitors are present which, as may be expected, are progressively weakened in effect by dilution as shown in Fig. 4. These inhibitors appear to be generally distributed in tissues as may be judged from Fig. 3 and may act in a similar way here on dephosphorylation as on deamination processes since vegetable adenylic acid in blood shows a somewhat similar curve on a much lower level; also in most of the tissues in Fig. 3 a dephosphorylation actually precedes the deamination (at least to a very large extent).

By shifting the *p*H of diluted blood towards 6·0–6·5 with citric buffer, the effect of the inhibitor is much weakened, the activity at 0·1 % nucleotide being 62 % of that at 1·0 %. The indication is that the inhibiting substance is an anion of a weak acid with *p*K value in the region of 7·0 and may be possibly a protein anion or anions. In voluntary muscle extracts such inhibitors have no appreciable effect in reducing the activity of the enzyme.

The question arises, however, how far do the normal CO_2 and bicarbonate concentrations in muscle affect the adenylic deaminase. As shown, in very weak dilutions (1/2000), *M*/40 bicarbonate and 38 mm. CO_2, the effect is very great. With stronger extract (1 in 40), 80 mm. CO_2 and *M*/40 bicarbonate the effect is much less, the deamination of 0·1 % nucleotide being 40 % of that of the 1 % solution. It may be presumed that with the very high enzyme concentrations in the tissue itself and a reaction around *p*H 7·0, the effect is not marked.

*The p*H *effect*

Adenylic acid. The effect is shown in Fig. 5 where the activity is given in terms of a 100 value at *p*H 7·0. The *p*H buffers used were maleic acid below *p*H 7·0 and bicarbonate above, the strengths being *ca.* *M*/20; a nucleotide concentration of 0·25 % was used in the mixture and blood dilutions of 1 in 40 (higher nucleotide concentrate would have been somewhat more satisfactory on the alkaline side). The slope of the curve on each side of *p*H 7·0 was determined and the values at 7·0 regarded as 100.

The curve does not show the sharp-peaked optimum close to 6·0 described previously by Schmidt [1928]. It may be noted at the same time that the question of specific buffer inhibition was not studied by this worker.

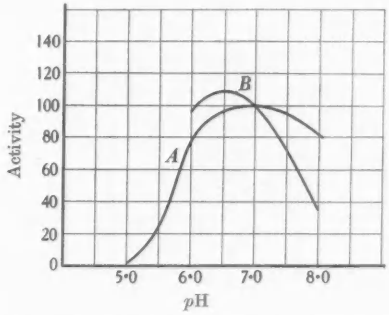

Fig. 5. Effect of *p*H on the deamination rate of m. adenylic acid (curve A) and of adenosine (B) in laked rabbit blood.

Adenosine. The adenosine curve was determined with phosphate buffers and 0·2–0·3 % adenosine. It does not differ appreciably from the results obtained using purified enzyme preparations [Schmidt, 1928].

The path of the deamination of muscle adenylic acid in tissues

The question here is what proportion of the deamination proceeds directly from the nucleotide or after an initial dephosphorylation. It may be considered for adenylic acid added to extracts, or for deaminations proceeding normally from the nucleotide complex. The study of the inorganic phosphate is not of much value for this purpose since phosphate may be transferred to other substances directly or after an initial liberation. We have used two deamination methods, however, whereby the question has been satisfactorily dealt with.

First method. In this method the deamination rate is examined after adding adenylic acid, after adding adenosine alone (enough to saturate the enzyme, which will occur after very small additions) and after adding adenylic acid plus adenosine. The difference between the deamination rates in the second and third mixtures will give the true or direct nucleotide deamination. Such a method will also give a measure of the true adenylic deaminase.

The method is not of immediate value in determining the path of normal deaminations owing to the much greater rate in general of adenosine deamination as compared with that of small added amounts of adenylic acid.

Second method. In this method the deamination rates of similar concentrations of yeast and muscle adenylic acids are studied in the same tissue extract or laked blood mixture.

Assuming that vegetable adenylic acid is not directly deaminated in animal tissues and that the enzymic dephosphorylation rates of both acids are the same (with acidic hydrolysis the yeast adenylic acid goes faster), we obtain the amount of direct deamination of the muscle nucleotide by the difference in deamination rates.

As regards the first point it has been shown by Schmidt [1928] that the purified enzyme from muscle is extremely specific and does not deaminate the vegetable nucleotide at an appreciable rate, and this we have also found for voluntary

muscle extract at room temperature (vide Table VI). In blood we have found that after elimination of dephosphorylation activity by dialysis the deamination rate of vegetable adenylic acid (in 1 % strength) falls almost to zero when examined over 24 hr., whereas that of the muscle adenylic acid remains practically unchanged. This is seen by comparing the two last columns of Table III.

Table III

		Phosphate μg./ml. P after		NH_3 μg./ml. N after	
	Mixture	2 hr.	20 hr.	2 hr.	20 hr
Undialysed	Blood control	34·3	46·0	1·8	5·3
	M. adenylic mixture (0·8 %)	29·9	30·7	124·0	215·0
	V. adenylic mixture (0·8 %)	41·8	64·0	9·3	32·3
Dialysed	Blood control	1·4	1·6	0·7	0·3
	M. adenylic mixture (0·8 %)	1·8	1·8	120·0	192·0
	V. adenylic mixture (0·8 %)	1·4	0·0	4·5	4·7

The blood dilution in mixture was 1 in 4·2, *p*H 8·0–8·3.

Table IV

Tissue	M. adenylic acid deaminated μg. N/ml./min.	V. adenylic acid deaminated μg. N/ml./min.	True nucleotide deaminated. II minus III μg. N/ml./min.
Blood	12·2	0·6	11·6
Auricle	13·3	2·7	10·6
Salivary gland	14·3	8·8	5·5
Jejunum	4·7	2·3	2·4
Stomach muscle	6·1	1·5	4·6

1 in 20 water extract of tissues were used. 1 vol. of extract plus 1 vol. of 2 % adenylic acid at *p*H 7·0. No other buffering used. Room temperature. The deamination rates are reckoned for the original tissue.

Table V

	Adenosine deaminated		Adenosine plus m. adenylic acid deaminated 0·16 % adenosine + 0·8 % m. adenylic acid in mixture	True nucleotide deaminated
Tissue	0·16 % μg. N/ml./min.	0·32 % μg. N/ml./min.	μg. N/ml./min.	μg. N/ml./min.
Blood	4·15	4·15	17·0	12·9
Auricle	6·3	6·7	17·1	10·6
Salivary gland	15·5	15·8	21·6	6·0
Jejunum	54·8	57·3	60·4	4·4
Stomach muscle	1·74	1·69	5·1	3·4

Similar conditions to those for Table IV, but maleic acid (*M*/20) used for buffering at *p*H 7·0.

As regards the second point—the equality of dephosphorylation of the two nucleotides—perhaps the best proof of this in the tissues generally is a comparison of the results from the two methods. This comparison is given in Tables IV and V for five tissues. The correspondence is quite good, especially when it is considered that analytical inaccuracies will have a big effect in the results from the salivary gland and jejunum extracts. Table III shows how little can be deduced from direct studies of the free phosphate.

With this second method we can examine the effect of adding amounts of each nucleotide to tissue extracts of a similar concentration to the nucleotide in the original tissue and so gain information as to the normal path.

For 11 tissues studied in this way (Table VI) voluntary muscle, the cerebral cortex, conducting nerve, auricle of heart and blood (under normal conditions) show direct deamination as the major path (75–90 %).

In the second group—which is sharply demarcated from the first—the deamination proceeds almost if not quite entirely by the indirect route. This group includes the ventricular muscle of the heart, stomach muscle, salivary gland, kidney, liver and jejunum. At high nucleotide concentrations apparently an appreciable fraction goes directly but as the nucleotide concentration is dropped from 1 to 0·1 % the NH_3 formations from the two nucleotides become the same. It is of interest to note how closely the deamination rates of the two substances approach each other in these different tissues. *From the composition of this second group we may infer that the general visceral deamination of adenine nucleotide is an indirect process.* The auricle of the heart is an interesting exception.

Table VI

I	II	III	IV	V	VI	VII	VIII
Tissue	Exp. no.	*p*H	Nucleotide concentration %	M. adenylic acid deaminated μg. N/ml./min. (×dilution)	V. adenylic acid deaminated μg. N/ml./min. (×dilution)	True nucleotide deaminated at 0·1 % μg. N/ml./min. (×dilution)	Ratio of VI to V %
Voluntary muscle	7	7·0	1·0	987·0	0·05		0·005
	7	7·0	0·1	886·0	0·05	886·0	0·005
Cerebral cortex	4	7·0	1·0	17·1	2·7		15·8
	2	7·0	0·1	3·22	0·55	2·87	10·9
Sciatic nerve	4	7·0	1·0	5·4	0·70		13·0
	2	7·0	0·1	2·59	0·61	1·98	23·6
Auricle	4	7·0	1·0	13·3	2·70		20·3
	2	7·0	0·1	2·93	0·72	2·21	24·8
Blood	3	7·4	1·0	4·25	0·48		11·3
	3	7·4	0·05	0·45	0·04	0·41	8·9
Blood	6	7·0	1·0	12·2	0·60		4·9
	6	7·0	0·1	0·27	—	< 0·27	
Salivary gland	4	7·0	1·0	14·3	8·8		61·6
	2	7·0	0·1	3·18	3·05	0·03	95·8
Kidney	1	7·0	1·0	6·50	3·68		56·8
	1	7·0	0·1	1·67	1·61	0·06	96·6
Liver	1	7·0	1·0	5·76	4·04		70·2
	1	7·0	0·1	1·13	1·07	0·06	94·6
Stomach muscle	4	7·0	1·0	6·10	1·50		24·6
	2	7·0	0·1	0·18	0·19	−0·01	105·5
Jejunum	4	7·0	1·0	4·70	2·30		48·9
	2	7·0	0·1	1·21	1·12	0·09	92·8
Ventricle of heart	1	7·0	1·0	4·72	4·78		101·5
			0·1			(0·00)	
Blood	5	8·3	0·1	0·028	0·027	0·01	96·4

The rabbit tissues were extracted with water, 1 part by weight to 20 vol. 1 vol. of extract was added to 1 vol. of nucleotide at *p*H 7·0. No other buffering was used except for blood and voluntary muscle, but the NH_3 formation was not allowed to exceed $N/5000$ strength, and no appreciable change of *p*H occurred. In the blood experiments 3, 6 and 5 CO_2/bicarbonate ($M/40$), maleic acid ($M/20$) and bicarbonate ($M/10$) were used, the blood dilution ranging from 1 in 5 to 1 in 8·8. Maleic acid buffering was used with the voluntary muscle extract. Room temperature.

For each experiment the tissues from two rabbits were used immediately after killing. After grinding the extract was usually allowed to stand for a few hours.

The above studies of deamination were carried out without further buffering than that provided by the nucleotide or the tissue extract. At the 1 % levels the nucleotide buffering is very appreciable. With the 0·1 % additions the NH_3 formation was not allowed to exceed about $N/5000$ with the result that no appreciable shift in pH occurred.

Seeing that in these 1 in 40 extracts the tissue Mg concentration has fallen considerably the effect of Mg addition was examined. No appreciable change from the above description was found.

The distribution of the deaminases in tissues

These were measured by the deamination rates of adenosine and adenylic acid under standard conditions, and the unit chosen was the μg. N/ml./min., the amount of enzyme being given per 1 g. or 1 ml. of the original tissue. A pH of 7·0 was selected as most suitable since it corresponds to the optimum formation of NH_3 from adenylic acid in tissues, is most representative of the actual tissue pH and is also close to the adenosine optimum (multiplying the adenosine results by 1·09 will give the value for the optimum pH).

1 in 20 water extracts of the tissues were made—grinding to a fine suspension with quartz sand—and an equal volume of substrate added. For adenylic acid

Table VII

Tissue	Adenosine deaminated	M. adenylic acid deaminated at 1 % (direct and indirect)
Alimentary:		
Appendix	59·7 (2)	8·3 (2)
Jejunum	52·6 (3)	6·6 (4)
Peyer's patches	36·8 (1)	12·1 (1)
Duodenum	29·6 (1)	—
Duodenum } mucosa	23·1 (2)	14·4 (2)
Jejunum } scrapings	21·1 (2)	11·6 (2)
Colon	10·7 (1)	—
Ileum	8·1 (2)	—
Caecum	6·5 (1)	—
Pyloric mucosa	2·8 (2)	2·2 (2)
Stomach muscle	2·0 (3)	5·1 (4)
Glandular:		
Spleen	40·2 (3)	14·8 (2)
Testicles	26·6 (3)	16·5 (2)
Salivary glands	19·7 (5)	9·8 (4)
Suprarenals	15·3 (3)	15·5 (2)
Pancreas	12·6 (3)	3·0 (2)
Thyroid	8·3 (2)	—
Kidney	7·7 (2)	5·6 (2)
Ovary	6·5 (2)	5·6 (1)
Liver	3·7 (3)	2·8 (3)
Pituitary	2·6 (2)	24·6 (2)
Muscular:		
Auricle	6·8 (6)	9·4 (2)
Diaphragm	2·5 (2)	108·0 (2)
Ventricle	2·3 (2)	2·8 (2)
Stomach muscle	2·0 (3)	5·1 (4)
Skeletal muscle	0·9 (4)	1145·0 (6)
Nervous:		
Spinal cord	14·7 (1)	14·8 (1)
Brain (whole)	8·6 (1)	12·2 (2)
Cerebral cortex	5·6 (1)	15·9 (3)
Pituitary	2·6 (2)	24·6 (2)
Sciatic nerve	0·8 (3)	4·8 (3)
Respiratory:		
Lungs	8·0 (3)	6·0 (2)
Circulatory:		
Auricle	6·8 (6)	9·4 (2)
Whole blood	5·9 (5)	14·0 (5)
Artery	4·1 (2)	2·2 (2)
Ventricle	2·3 (2)	2·8 (2)
Plasma	0·04 (2)	0·04 (2)
Miscellaneous:		
Embryonic tissue	10·6 (2)	10·7 (2)
Bone marrow	8·3 (2)	9·7 (2)
Uterus	3·7 (1)	6·5 (1)
Skin	0·0 (1)	0·0 (1)
Bone	0·0 (1)	0·0 (1)

The units in which the deamination is expressed are μg. N/ml./min. for the original tissue. Tissues ground with quartz in 20 vol. water. To 1 vol. extract 1 vol. of nucleoside plus 0·5 vol. buffer (phosphate $M/20$) or 1 vol. extract plus 1 vol. of 2 % nucleotide. pH 7·0. Room temperature.

this consisted of 2 % nucleotide which had been neutralized to *p*H 7·0 with NaOH and for adenosine 0·5 % adenosine buffered with phosphate at *p*H 7·0 to make *M*/20 in the mixture. No buffer except that of the nucleotide itself in 1 % strength in the mixture was used in studying the distribution of the adenylic deaminase.

The unexpected behaviour of adenylic deaminase in tissues as regards the points already considered led us to examine the efficiency of extraction of the enzyme under various conditions. In our experiments the efficiency of extraction is somewhat variable even under strictly standardized conditions, though this may be attributable to varying distribution throughout the tissue. Using pure water, 0·9 % NaCl and 0·9 % NaCl plus 0·05 % KCl we obtained a ratio of 1·0 : 1·2 : 1·6 in the amounts of enzyme action observed in a few experiments. These extractions were made from the same muscle finely cut and mixed, some of which was weighed out and ground with quartz and the fluid to make 1 in 20 extracts. The inclusion of Mg and Ca with the 0·9 % NaCl caused a decrease, which also resulted from the inclusion of some bicarbonate in the extract medium. From this it will appear that saline would have been a more efficient extractor than water and there is also a case for supposing K addition to act better than Na alone, though to decide this many more experiments would be necessary. Since for reasons already given the amount of the deaminase can be given only in a comparative way, the water extract was considered sufficiently serviceable.

Table VII gives a summary of the experiments performed on 36 tissues of the rabbit, the results being considered in the subsequent discussion. The adenylic deamination given in Table VII indicates the sum of both direct and indirect formations at 1 % concentration, from which, as already considered, we must distinguish the pure nucleotide deamination and the effective deamination rate in tissues.

Discussion

The distribution of adenylic acid and adenosine deaminases in the tissues of the rabbit. The distribution of the adenosine deaminase may be roughly divided into a digestive, a glandular, a nervous and a muscular type and in that order with respect to enzyme strength. The jejunum and duodenum were found to contain 53 and 30 units respectively *while the appendix showed the highest of any tissue examined*, namely 60 units. In each case all the intestinal coats were taken together, but scrapings from the mucosa of the duodenum and jejunum gave also no increase over these figures, but rather a decrease.

An explanation of the high appendix concentration compared with that in the ileum, caecum and colon may be given by considering that in the rabbit—and possibly in other herbivora—the caecum serves as a second stomach in which the cellulose walls of cells are digested by special bacteria, further nucleic acid material being liberated. The appendix may then be represented as playing the same role as the duodenum and jejunum to the main organ with respect to the further treatment of nucleic acid.

In the glandular distribution the spleen comes highest, but this tissue is exceptional with regard to the adenylpyrophosphate liberated therein from the red corpuscles. The special glandular activity is shown rather by the testicles, salivary glands, suprarenal and the pancreas, which gave from 27 to 13 units. Of the eleven types of glandular tissue examined the liver gave the second lowest with 4 units and the pituitary lowest with 3 (the pituitary contained relatively a very high proportion of adenylic acid deaminase, namely 25 units).

The nervous distribution of adenosine deaminase is somewhat similar to the glandular, being perhaps a little lower. The difference here is in the compara-

tively higher amount of adenylic deaminase, the ratio being about 3 to 1 in the cerebral cortex and 6 to 1 in the conducting nerve (the latter containing only 0·8 units of adenosine deaminase).

Muscle tissue in general contained the lowest amounts, with the auricle of the heart highest in the group, having 6·8 units, and voluntary muscle lowest with 0·9. The muscle of the stomach wall had 2·0 units and the ventricle of the heart 2·3.

Concerning the distribution of true adenylic acid deaminase, voluntary muscle is very exceptional with an average of 1145 units. A long way after this we have nervous tissue, e.g. cerebral cortex, with 15·8 units (vide Table VI) but only an effective deamination rate of less than 2·9. Conducting nerve also has a high ratio of adenylic acid deaminase to that of adenosine (6 : 1). Blood was found to contain 14 units and the auricle of the heart 9–11. Apart from these tissues the amount of true adenylic acid deaminase found elsewhere was low and its effective action practically zero. Contrary to our first observations plasma was found to contain a very small and variable amount of the true nucleotide deaminase, but at any possible normal concentrations its activity is only a few % of the adenosine deaminase therein (0·05 unit).

The significance of the adenylic acid and adenosine deamination

That NH_3 formation is coincident with activity in tissues is a point that, as is well known, has been emphasized by Embden and others. Adenylic acid was shown by Embden to be the precursor of this NH_3 in voluntary muscle. Later it was found by Lohmann [1929] that adenylic acid was itself a stage in the breakdown of adenylpyrophosphate, the role of which in phosphate transference and the glycolytic cycle was soon recognized. It was subsequently considered—in particular by Parnas—that the deamination of the adenylic acid formed on dephosphorylation and such as escaped resynthesis was merely a detoxication process without any special relation to muscular activity. Certain findings in the present paper point directly against this view and may be summarized as follows.

(1) An average of about 40 times more true adenylic acid deaminase exists in voluntary muscle than in any other tissue examined, but the effective deamination rate is greater by about 500–1000 times or more, owing to the absence therefrom—or at least the ineffective action—of specific inhibitors of the enzyme.

(2) Though quite considerable amounts of the deaminase exist in the red corpuscle and other tissues, the action of the enzyme is reduced to a few % or less of its full action by special inhibition (in addition to that produced by the CO_2 and bicarbonate system). In this way adenylic acid can be said to be conserved and presumably as a physiological process. Yet adenylic acid is freely permeable across the membrane of the red corpuscle and will therefore escape into the plasma. We have demonstrated this permeability to adenylic acid added both to oxalated and to heparinized blood and examined immediately after shedding, and also a similar permeability for voluntary muscle.

(3) An adenine nucleotide apparently already exists in plasma and its pharmacological action cannot be considered as markedly less than that of muscle adenylic acid [Bennet & Drury, 1931].

(4) The deamination of adenine nucleotide in the duodenum or jejunum is not direct but occurs after dephosphorylation. Here the total effective deamination of the nucleotide for normal concentrations is only a minute fraction of that in skeletal muscle—probably considerably less than 1 %—yet we may suppose that across the intestinal wall there pass amounts of nucleotide at least as great

as the adenylic acid set free momentarily and escaping re-esterification in voluntary muscle.

A point which has appeared conclusive to Lohmann [1935] against the special role of adenylic deaminase is the absence—as he found—of this enzyme in the muscle of the crab—including the claw muscle. We have investigated this point in turn and found for the claw muscles from four crabs a mean value of 40 units of the deaminase. While this amounts only to about 4 % of that in the rabbit's leg muscle, it is still very appreciable and higher than that found in any of the other tissues of the rabbit. Considering the many factors discussed above which inhibit the action of this enzyme it is not surprising that its absence is occasionally and erroneously concluded.

Direct nucleotide deamination can also occur normally in nerve tissue, the auricle of the heart and in red corpuscles though these have no obvious relationship. It may be noted that the conservation of adenylic acid in general tissues by the action of the specific inhibitors and CO_2 (or bicarbonate) combined with its permeability (as we may deduce from muscle and red corpuscles) may lead to the activity of this substance as a local vasodilator.

Concerning the deamination of adenosine, this may be firstly considered as merely a process in the metabolism of surplus nucleic acid, and in the intestinal wall all the normal deamination of adenine nucleotide derives from this substance. Besides this, the widespread occurrence of adenosine deaminase in tissues and in blood may be regarded from two aspects. Adenosine may be looked upon as an accidental formation from free adenylic acid, set free in turn from A.T.P. in the glycolytic process, and that when formed it is detoxicated by deamination. Adenosine is apparently the most active substance pharmacologically of its class, and that it should be rapidly deaminated in the intestinal wall may be linked with the detoxication view. We may consider too that adenosine deamination in the general tissues is a physiological mechanism for obtaining a ready supply of NH_3—which they cannot obtain directly from blood —and that the role of this NH_3 may be of a similar kind, though much more slowly formed, than the NH_3 in voluntary muscle. What this role may be is so far obscure, but various possibilities can be suggested such as permeability changes caused thereby, requirement for local synthesis of amino compounds, local neutralizations etc.

Summary

1. The relation of adenylic acid deaminase to substrate concentration is affected by specific buffer inhibition and also by special inhibitors in tissues. These appear to act by displacing the adenylic acid from an adsorbing surface. Among the buffers so acting are CO_2 and bicarbonate, phosphate and veronal. Maleic and citric acids do not act in this way. The effect of the inhibition is to simulate a linear relation of action to substrate up to very high concentrations (about 1 % or more). The special tissue inhibitors are either absent from or ineffective in voluntary muscle. Dilution of blood—by lessening the concentration of the inhibitors can very greatly increase the deamination of adenylic acid per ml. blood.

Similar inhibitions of the action of adenosine deaminase have not been found.

2. Methods for determining the path of the deamination of adenine nucleotide are given. It is shown that (apart from voluntary muscle) nerve tissue, the auricular muscle of the heart and red corpuscles deaminate adenylic acid normally in a direct way, but the kidney, liver, intestine, smooth muscle, ventricular muscle of heart and blood after loss of CO_2 deaminate it only after an initial dephosphorylation.

3. The distribution of adenosine and adenylic acid deaminases has been studied for 36 tissues of the rabbit. The amount of adenylic acid deaminase in voluntary muscle has a mean value 40 times greater than that in any other tissue examined, but its effective action on possible normal concentrations is greater by about 500–1000 times or more. Through the inhibition of the adenylic acid deaminase in blood, although there is more of this than the corresponding adenosine enzyme, the action on minute amounts is less than 1 % of that on the nucleoside. Even those tissues, apart from voluntary muscle, which normally deaminate the nucleotide directly are less effective deaminators of small amounts of adenylic acid than of adenosine.

The highest concentration of adenosine deaminase was found in the vermiform appendix and somewhat lesser amounts in the duodenum and jejunum.

4. Contrary to Lohmann's finding [1935], adenylic acid deaminase in very appreciable amounts was found in the claw muscles of the crab.

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LX. THE INFLUENCE OF LOW AND HIGH PLANES OF NUTRITION ON THE COMPOSITION AND SYNTHESIS OF FAT IN THE PIG

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The work described in this communication deals with the determination of the component acids in the depot fats of pigs which had received controlled diets at different levels of nutrition. The primary object was to ascertain how far the quantitative proportions of the various component acids varied in relation to the feeding level of the animal. It was also found possible to make a rough quantitative comparison of the amounts of each fatty acid (*a*) ingested by the animal, (*b*) deposited in the animal in the form of fat, and therefrom to deduce the amount and nature of fat synthesized in the animal, and also to compare the rates of deposition of fat in animals which had been given different rations.

An investigation has recently been in progress at the Animal Nutrition Institute of the School of Agriculture, Cambridge, designed to determine the effect of rate of growth on the proportions of the different parts and tissues in the pig. The physiological and anatomical results of this investigation are in course of publication by Dr C. P. McMeekan. By the courtesy of Dr J. Hammond, specimens of the back and perinephric fats, in sufficient amounts for detailed component acid analyses to be made, were placed at our disposal, together with details of the diets received by the pigs and of the weights of their fatty tissues at the time of slaughter. We are informed by Dr Hammond that the plan of feeding was as follows.

Thirty pigs of unusually homogeneous (inbred) stock were fed on the same diet in different amounts. Up to the age of 16 weeks the pigs were in two groups:

(*a*) On high plane of nutrition, final weight 120–132 lb. (average 126 lb.).
(*b*) On low plane of nutrition, final weight 32–37 lb. (average 34 lb.).

Subsequently half of the pigs in each group continued to receive food at the same plane as during the first 16 weeks, whilst the other half were transferred to the alternative level, those on the high plane now receiving the low plane diet, and conversely. The restriction of the ration was that required to make the pigs grow according to pre-arranged growth curves. All these animals were grown to 200 lb. weight before slaughtering.

Details of the rations supplied to the animals were as follows:

	High plane	Low plane
Ration while on sow ...	Free access to sow and to dry meal mixture no. 1; separated milk *ad lib.*	Restricted access to sow and to dry meal mixture no. 1 and to separated milk
Ration from weaning to 16 weeks	1 gal. separated milk per pig daily and meal mixture no. 1 *ad lib.*	½ gal. separated milk per pig daily and restricted ration meal mixture no. 1
Ration from 16 weeks onwards	1 gal. separated milk per pig daily and meal mixture no. 2 *ad lib.*	½ gal. separated milk per pig daily and restricted ration meal mixture no. 2

Composition of meal mixtures

No. 1	%	No. 2	%
Dried separated milk	20	Barley meal	30
White fish meal	30	White fish meal	30
Wheat middlings	30	Flaked meal	30
Flaked meal	20	Wheat middlings	10

The first group of pigs had passed the 16 weeks period before it was decided to reserve sufficient amounts of the back and perinephric fats for the present work, but these specimens were obtained from the final animals killed at 200 lb. weight (pigs nos. 72, 73, 74, 82); a duplicate experiment up to 16 weeks was carried out later from which samples of fat (pigs nos. 138 and 139) were supplied for our analyses.

The weights of fat deposited in the various tissues of each of the animals are summarized in Table I from data kindly supplied to us by Dr C. P. McMeekan (working with Dr Hammond).

Table I. *Total weight, etc., of fat in tissues*

Pig	73 (hog)	74 (gilt)	72 (hog)	82 (gilt)	138 (gilt)	139 (gilt)
Planes of diet	Low-High	High-High	High-Low	Low-Low	High (16 weeks)	Low (16 weeks)
Age (days)	212	181	212	339	112	112
Farm weight (lb.) ...	204	198	198	197	132	37
Fat in tissues (kg.):						
Subcutaneous	21·69	16·46	15·41	9·80	8·73	0·31
Perinephric and kidney	2·00	1·46	1·50	1·04	0·74	0·02
Intermuscular	6·20	5·96	4·68	4·33	3·27	0·16
Mesenteric	0·95	1·08	0·79	0·93	0·57	0·05
Caul	0·24	0·15	0·11	0·12	0·07	—
Total fat (kg.)	31·08	25·11	22·49	16·22	13·38	0·54
Rate of deposition of fat (g. per diem):						
Subcutaneous	102	91	73	29	78	3
Perinephric	9	8	7	3	7	0·2
Total body fat	147	139	106	48	119	5

Apart from insufficiency of material in certain instances, it was out of the question to carry out detailed fatty acid analyses on each of the 34 depot fats in Table I. Large specimens (1000–1200 g.) of the outer and inner back fats, and of the perinephric fats, were however prepared by extracting the fatty tissues with acetone [cf. Banks & Hilditch, 1932]. After a preliminary analysis of both inner and outer back fats[1] from pig no. 74, which showed differences between the two fats similar to those previously noted [Banks & Hilditch, 1932; Dean & Hilditch, 1933, 2], it was decided to confine detailed examination for the time

[1] I.e. the layers of fat on the inner and outer side of the well-marked dividing line of connective tissue in the subcutaneous fatty layer lying on the thorax and loin of the pig.

being to the outer back fats. This choice was mainly determined by the fact that the unsaturated acids (in which we were particularly interested from the standpoint of keeping qualities of the fat) were known to be present in greater proportions in this section of the fats than in the inner back fat or the perinephric fat. The general characteristics of all the fats which were extracted in quantity are, however, given in Table II.

Table II

Pig no.	Planes of diet	Perinephric fat I.V.	Inner back fat I.V.	Outer back fat I.V.	Outer back fat Sap. equiv.	Outer back fat Unsap. (%)
73 (hog)	Low-High	52·4	55·6	58·5	288·8	0·2
74 (gilt)	High-High	49·5	54·4*	59·8	285·9	0·2
72 (hog)	High-Low	53·3	57·2	63·3	288·0	0·1
82 (gilt)	Low-Low	57·4	60·4	65·5	286·5	0·1
138 (gilt)	High (16 weeks)	51·0	56·0	60·5	†	†
139 (gilt)	Low (16 weeks)	—	‡	‡	‡	‡

* Sap. equiv. 285·4, unsaponifiable 0·1%.
† No. 138, composite back fat, I.V. 58·0, sap. equiv. 284·9, unsaponifiable 0·1%.
‡ No. 139, composite back fat, I.V. 62·9, sap. equiv. 287·8, unsaponifiable 0·2%.

Experimental

Determination of component fatty acids followed the lines which have been described in recent communications from this laboratory: (*a*) separation of the mixed fatty acids from about 200 g. of each fat into "solid" and "liquid" acids by the different solubilities of the lead salts in alcohol, and (*b*) fractional distillation at low pressure of the methyl esters of each group of fatty acids. As a preliminary to the examination of a typical depot fat from each of the six experimental animals, both the inner and outer back fats of the pig which had been fed throughout on the "high" plane of nutrition were studied.

In the case of the inner back fat, separate portions of the esters of the "liquid" acids were distilled by the simple "Willstätter bulb" apparatus, and through an electrically heated and packed column which gives sharper separation of some of the minor components [Longenecker, 1937; Hilditch & Longenecker, 1937]. This showed that the data obtained with the simpler fractionation apparatus, when calculated in terms of all the acids shown to be present by use of the more elaborate column, give results which accord with those from the latter, when dealing with the comparatively simple mixture of fatty acids present in pig and similar depot fats (Table III). Nevertheless the electrically heated column, although it involves a longer period of distillation and also more practice in operation in order to obtain adequate control, gives extremely sharp separation of the successive ester fractions. In the main part of the work the esters of "solid" acids were distilled from a Willstätter bulb, and those of the "liquid" acids through the electrically heated column.

The proportions of the component acids of the inner (Table III) and outer (Table IV) back fats of pig no. 74 have the same relationship to each other as in previously examined specimens of inner and outer back fats from individual pigs [Banks & Hilditch, 1932; Dean & Hilditch, 1933, 2]. The minor component acids are not significantly different, the percentage of palmitic acid is slightly lower in the outer than in the inner back fat, and the chief difference is an increase in the oleic acid content of the outer fat, mainly at the expense of stearic acid.

Table III. *Component acids of inner back fat of pig no. 74*

	"Solid" acids 44·8%	"Liquid" acids 55·2%		Total fatty acids (excluding unsaponifiable matter) % (wt.)		% (mol.)	
Fractionation apparatus ...	W.	W.	E.H.P.	W.	E.H.P.	W.	E.H.P.
Myristic acid	0·03	0·47	0·99	0·5	1·0	0·6	1·2
Palmitic acid	27·41	3·62	2·73	31·1	30·1	33·1	32·2
Stearic acid	16·14	—	—	16·2	16·2	15·5	15·5
Tetradecenoic acid	—	—	0·28	—	0·3	—	0·3
Hexadecenoic acid	—	2·88	2·69	2·9	2·7	3·1	2·9
Oleic acid	1·19	39·73	39·65	41·0	40·9	39·7	39·6
Linoleic acid	—	7·10	7·10	7·1	7·1	6·9	6·9
Unsaturated C_{20-22} acids	—	1·27	1·67	1·3	1·7	1·1	1·4
Unsaponifiable	0·03	0·13	0·09	—	—	—	—

W. = esters distilled from a Willstätter bulb.
E.H.P. = "liquid" esters distilled through the electrically heated and packed column.

Constitution of the hexadecenoic acid of pig depot fats. Hilditch & Shorland [1937] found evidence, contrary to previous observations, of the presence of perhaps 3–4 % of hexadecenoic acid in pig depot fats. The present series of analyses show that pigs of about 200 lb. live weight contain 2–3 % of this acid in the mixed acids of the back fats, and the opportunity was taken to ascertain its structure.

A number of the ester fractions, with equivalents ranging from 260 to 280 and I.V. from 40 to 70, obtained from methyl esters of the "liquid" acids of the pig back fats, were united, and their mixed acids submitted to the lead salt-alcohol separation to remove as much palmitic acid as possible. After reconversion of the "liquid" acids thus obtained into methyl esters (28·4 g.), a further distillation through the electrically heated column gave a fraction (11·6 g., sap. equiv. 268·0, I.V. 86·7, CNS value 87·1) which appeared to be substantially methyl hexadecenoate (sap. equiv. 268·0, I.V. 94·8) with minor proportions of methyl palmitate and, perhaps, myristate. (It will be noted that methyl $\Delta^{9:10}$-hexadecenoate reacts normally as a monoethenoid derivative with Kaufmann's thiocyanogen reagent, the thiocyanogen and iodine values being identical.)

Oxidation of this ester in acetone solution with powdered $KMnO_4$ gave azelaic acid (M.P. and mixed M.P. 101°) and heptanoic acid (Et ester, sap. equiv.: found 154·3, calc. 158·0). The acid in pig depot fat, like that of ox depot fat [Hilditch & Longenecker, 1937], is therefore $\Delta^{9:10}$-hexadecenoic acid. This was further confirmed by the production, on oxidation of a dilute alkaline solution of the acid with $KMnO_4$, of 9:10-dihydroxypalmitic acid (M.P. and mixed M.P., 125°).

Nature of the highly unsaturated C_{20-22} acids present in pig depot fats. Brown & Deck [1930] showed that pig fats contained 0·3–0·4 % of highly unsaturated acids, estimated as arachidonic acid, $C_{20}H_{32}O_2$, from the yield of polybromides from the distilled esters of the lard fatty acids (employing the observed yield of polybromides from methyl arachidonate, 77·6[1]). The approximate proportion of C_{20-22} acids, estimated from the equivalents of the highest-boiling fractions of the methyl esters, was found to be somewhat larger (*ca.* 1 %), by Banks & Hilditch [1932] and Dean & Hilditch [1933, 2], whilst in the present series it was uniformly found to be about 2 %.

[1] Subsequently Ault & Brown [1934] gave the yield of insoluble polybromo-adducts from pure methyl arachidonate as 86·5 % of the weight of the ester.

In the present experiments, the polybromo-adducts from accumulated high-boiling fractions from the pig back fats were compared with those from the oil extracted from the fish meal used in the diet of the animals. The polybromo-additive products from each source were debrominated in pyridine solution with zinc [Kaufmann & Mestern, 1936]; the respective products had the following characteristics:

	Acids from debrominated pig fat polybromo-adducts	Acids from debrominated fish meal oil polybromo-adducts
Appearance	Pasty solid	Liquid
Yield from polybromo-adducts	33·8%	27·6%
I.V.	314	336
CNS value	94·5	178

These figures support Brown's observation [1931] that the deposited C_{20-22} acids differ from those ingested as fish oil, and are less unsaturated than the latter; but they do not necessarily imply that arachidonic acid is the only acid concerned. The equivalents of the residual esters left after fractionating the esters of the "liquid" acids (after correcting for any unsaponifiable matter present) were never below that of the ester of a C_{20} acid, and in one case the figure was considerably higher. Since these residual esters must in all cases have still contained some oleate, it follows that some quantity of esters of C_{22} acids was also present.

Component fatty acids of the outer back fats of pigs killed at 200 lb. live weight

About 300 g. of the outer layer of the back fat of each of the four animals fattened to 200 lb. live weight at different planes of nutrition were converted into the mixed fatty acids, which were then resolved into "solid" and "liquid" acids as described earlier. The methyl esters of the "solid" acids were fractionated from a Willstätter bulb, and those of the "liquid" acids through the electrically heated column. The lengthy detailed fractionation data are unnecessary for the subsequent discussion, and only the final results of the analyses need be recorded here. In Table IV the proportions of the component acids are given in the form of both weight and molar percentages.

Table IV. *Component acids of outer back fats (pigs killed at 200 lb. wt.)*

Pig no.	73		74		72		82	
Sex	Hog		Gilt		Hog		Gilt	
Planes of nutrition*	Low-High		High-High		High-Low		Low-Low	
	% wt.	% mol.	% wt.	% mol.	% wt.	% mol.	% wt.	% mol.
Myristic acid	1·1†	1·4†	1·3	1·6	0·7†	0·8†	0·8†	1·1†
Palmitic acid	28·2	30·2	28·3	30·2	25·3	27·2	25·9	27·8
Stearic acid	13·5	13·0	11·9	11·5	13·1	12·7	12·2	11·8
Tetradecenoic acid	0·2	0·2	0·2	0·2	0·1	0·1	0·2	0·2
Hexadecenoic acid	2·4	2·6	2·7	2·9	2·0	2·1	2·0	2·1
Oleic acid	47·0	45·4	47·5	46·0	51·0	49·7	48·1	46·8
Linoleic acid	5·2	5·1	6·0	5·8	5·3	5·2	7·8	7·6
Unsaturated C_{20-22} acids	2·4	2·1	2·1	1·8	2·5	2·2	3·0	2·6

* I.e. up to age of 16 weeks, and from 16 weeks to slaughter.
† Includes traces of a lower saturated (lauric?) acid.

Component fatty acids of composite back fats of pigs killed at age of 16 weeks

The determinations were carried out as in the preceding cases, but only about 40 g. were available of the total back fat of the pig fed on the low plane of nutrition. For this reason a specimen of the composite (inner + outer) back fat of each animal was examined in these cases. The final results are given in Table V.

Table V. *Component acids of composite back fats (pigs 16 weeks old)*

Pig no.	138		139	
Sex	Gilt		Gilt	
Plane of nutrition	High		Low	
	% (wt.)	% (mol.)	% (wt.)	% (mol.)
Myristic acid	1·0*	1·2*	1·3	1·5
Palmitic acid	29·8	31·7	28·1	29·9
Stearic acid	12·7	12·2	11·8	11·3
Tetradecenoic acid	0·2	0·2	Trace	Trace
Hexadecenoic acid	3·5	3·8	4·8	5·2
Oleic acid	47·8	46·2	42·9	41·5
Linoleic acid	3·1	3·1	8·2	8·0
Unsaturated C_{20-22} acids	1·9	1·6	2·9	2·6

* Includes traces of a lower saturated (lauric?) acid.

Discussion

It is well recognized that diets rich in fat cause pigs to deposit much of the ingested fat and that, for example, the inclusion of liquid fats rich in oleic and linoleic glycerides, such as cottonseed, maize or groundnut oils, in the ration tends towards the production of soft and oily pig fats [cf. Ellis *et al.* 1926, 1, 2; 1931; 1938]. In the present experiments we are less concerned with the problem of ingested fat than of that synthesized by the animal, since the total fat content of the diets was very low. The fat content of the meal mixtures was 2·2 %, whilst that of the separated milk (which formed the major constituent of all the feeds) was of the order of 0·1 %; the sow's milk taken before weaning contained about 4·5 % of fat. The percentage of fat in the whole of the food taken by the pigs varied somewhat according to the different ratios of separated milk and meals taken in the various experiments, but it never exceeded 1 % of the total diet, the extreme limits being 0·5 % at the "low-low" and 1·0 % at the "high-high" planes of nutrition. Any significant changes in the composition of the depot fats must therefore result almost wholly from differences in the amount of food given to the animal, and not from any sensible alteration in the amount of fat ingested.

We may take in the first place differences in the proportions of the various component acids (Tables IV and V), although these are less notable in percentage form than when the total amount of each component deposited as fat by the animal is considered (cf. below, Table VII). In the group of outer back fats, there is a slight but apparently definite increase in oleic acid content in the case of the 200 lb. weight animals which were fed from 16 weeks onwards on a restricted diet. Parallel with this, the palmitic acid contents in these instances are somewhat lower than in the outer back fats of pigs fattened on the higher plane of nutrition; and this also holds for the feeding at the two dietary levels during the first 16 weeks. In all cases, the variation is within the limits of

30 ± 3 % (mol.) which have been found to be the characteristic palmitic acid content of the acids of herbivorous animal depot fats [Hilditch & Longenecker, 1937]. The stearic acid contents of all six outer back fats showed little variation.

Of the minor components, it will be seen that only traces of saturated acids of lower molecular weight than myristic acid are present. Hexadecenoic (and traces of tetradecenoic) acids are fairly constant at 2·0–2·7 % in the fats of the 200 lb. weight animals, but somewhat higher at the 16 weeks' stage, irrespective of the plane of nutrition. The unsaturated C_{20-22} acids are more prominent than has been observed in previous cases; and in this connexion it should be noted that the proportion of fish meal, 10–13 % of the total diets, was fairly considerable. Woodman *et al.* [1937] state that inclusion of 5 % of fish meal in the ration up to the time of slaughtering involves a risk of producing a fishy taint in bacon.

The most variable of the minor constituents is linoleic acid. In fats from 200 lb. weight animals fattened on the high plane diet, this formed about 5–6 % of the fatty acids, whilst at 16 weeks (high plane) it was only 3 %; but it reached 8 % of the total outer back fatty acids of the animals fed on the restricted ration, either at 16 weeks or throughout until the pig had reached 200 lb. live weight. This variation is significant in regard to the relative softness of the depot fats (and, possibly, their keeping quality; although in the latter respect other factors may also come into play). Differences in linoleic acid content, though small numerically, can have an important effect on the glyceride structure of the fat [Green & Hilditch, 1938]: linoleic acid, as a minor component, will not be present more than once in any triglyceride molecule, whereas oleic acid, the most prominent component, will be present in the majority of the triglyceride molecules. Hence any linoleic acid is almost certain to be present as a linoleo-oleo-glyceride, and of course glycerides containing two unsaturated acyl groups are liquid at the ordinary temperature. Consequently, whilst a content of 5 % of linoleic acid connotes about 15 % of corresponding liquid glycerides, 8 % of linoleic acid involves the presence of about 24 % of liquid glycerides—nearly 10 % more of the fat being of a type of lower melting point in consequence of an increase of 3 % in the linoleic acid percentage. The increases observed, at low planes of nutrition, in the linoleic, and also the oleic, acid contents of the depot fats are clearly the cause of the already known fact [Ellis *et al.* 1925; 1930; Robison, 1931; Callow, 1937] that rapid growth of pigs (i.e. increased rate of fat deposition) leads to the production of firmer fat.

Apart from increased tendency to softness of fat, the uneconomic rate of production of fat at low as compared with higher planes of nutrition is clearly evident from the data in Table I.

The variations in the rate of fat deposition, and also in the mean unsaturation of the fats (so far as can be judged (Table II) from I.V. alone), are parallel in all the depots of the animal. The most rapid rates of production, and more saturated (i.e. firmer) fats, result when the diet after the first 16 weeks is on a high plane of nutrition. Abundant diet is more important during these latter stages of growth than during the first 16 weeks of life. As a matter of fact, the most rapid production, but not quite the firmest type, of fat resulted from restricted diet up to 16 weeks, followed by the high plane of nutrition for the remainder of the life of the animal. A "low plane" diet up to 16 weeks followed by "high plane", again, gives much better results both as regards rate of fat production and relative saturation of the fat than the alternative of "high plane" diet up to 16 weeks and then a restricted diet to 200 lb. weight.

It should be noted that these conclusions refer only to fat formation in the animal, and take no account of bone and muscle production. Thus, although the fat is softer and produced more slowly in the animal fed on the "high-low" planes of nutrition than in that fed on the "low-high" planes, the former animal is of more desirable conformation as a whole—long and lean—than the latter which is of the short, fat type.

The effects of restricted rations throughout the whole life of the pig are seen to be very uneconomical in the long period taken to reach 200 lb. live weight, the excessive consumption of food required to produce a given increase in body weight, and in the relatively unsaturated and soft character of the resulting depot fats.

The component acid data discussed above may be utilized to yield some information of an approximate order as to the amount of each individual fatty acid deposited by the pig, and to compare these figures with the corresponding amounts of the fatty acids consumed by each animal in its diet. Obviously, a number of approximations and estimates have to be made in order to obtain all the necessary data, but we have satisfied ourselves that any errors thereby introduced are insufficient to alter the general conclusions to be drawn later from this comparison. The computation of individual acids in the fats of the diet is that most liable to errors in estimation, but the total quantity of fat ingested by any animal is so much less than that deposited in the animal, that wider variations from our estimates of the component acids present than are at all likely would have only minor effects on the ultimate comparison between ingested and deposited fats. The following methods have been used in these calculations.

Component acids ingested as fat in the diet. The amount of each individual fatty acid combined as fat in 100 parts of each of the constituents of the food given to the pigs has been calculated with the results shown in Table VI. The component acid data used for the dietary fats have been taken from the following sources:

Sow's milk fat. The figures are from a partial analysis (not using ester-fractionation) [Laxa, 1931].

Cow's milk fat. The data obtained by ester-fractionation for a typical cow's milk fat of similar I.V. by Dean & Hilditch [1933, 1] have been used, but modified to include the 5 % of lower unsaturated acids now known to be present [Hilditch & Thompson, 1936; Longenecker & Hilditch, 1938].

White fish meal oil. The component acids are taken from analyses by Lovern [1937] for halibut and turbot flesh fats (no others except herring being available). If the C_{20-22} acid contents in other fish flesh fats should be somewhat lower, such difference will not affect the conclusions drawn from Table VII (below).

Wheatmeal, maize and barley oils. The fatty acid data are based on recent determinations by modern methods respectively by Sullivan & Bailey [1936], Baughman & Jamieson [1921], and Täufel & Rusch [1929]. The I.V. of the meal oil sdiffered somewhat from those of the oils analysed by these workers, i.e. the proportions of oleic and linoleic acids are not exactly those given. It is hardly legitimate, in the case of these seed fats, to adjust the proportions of these acids according to the I.V. of the fats (*vide infra*), but here again alternative calculations have shown that the differences thereby introduced have no significance in regard to the final comparison. In accordance with recent observations [Hilditch & Jasperson, 1938], however, 1 % of hexadecenoic acid has been allowed in each case.

Component acids deposited as fat in the pigs. The data in each case depend upon our determinations of the acids in the outer back fats. Since it is now well

Table VI. *Approximate amounts of fatty acids present as fat in the dietary constituents*

Fatty acids	Sow's milk %	Separated milk %	Meal mixtures No. 1 %	Meal mixtures No. 2 %
Saturated:				
Below C_{14}	0·04	0·011	0·02	—
Myristic	0·13	0·010	0·05	0·03
Palmitic	1·21	0·024	0·27	0·22
Stearic	—	0·007	0·06	0·05
Unsaturated:				
C_{16} (and C_{14})	—	0·005	0·06	0·05
Oleic	2·94	0·034	0·58	0·63
Linoleic (and linolenic)	—	0·004	0·79	0·89
C_{20-22}	—	—	0·24	0·24
	4·32	0·095	2·07	2·11

established that, within the fats of a single animal, or indeed over wider ranges of pig, ox and sheep depot fats, difference in total unsaturation is very largely conditioned by respective differences in the contents of oleic and stearic acids [cf. Tables III and IV; Banks & Hilditch, 1931; 1932; Dean & Hilditch, 1933, 2], it is quite safe in these cases, for small differences in I.V., to adjust the stearic and oleic acid contents determined on one of the depot fats of the animal concerned. This has been done in the case of each pig in the present series. The I.V. of the inner and outer back fats and the perinephric fats are given in Table II; the intermuscular fats were assumed to have similar I.V. to the inner back fats, and the mesenteric and caul fats to have similar I.V. to the perinephric fats. From the total weights of fat in each tissue (Table I) the individual acids deposited as fat were then calculated.

Table VII shows the weights (kg.) of each individual acid or group of acids thus calculated to have been (*a*) received as fat in the food or (*b*) deposited as fat

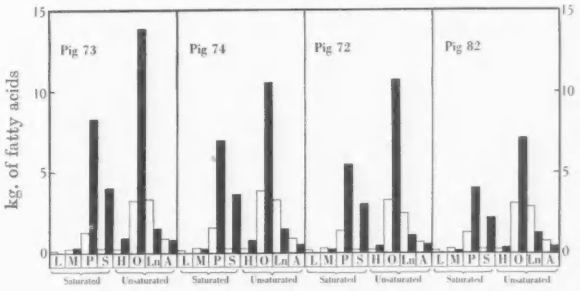

Fig. 1. Ingestion and deposition of fatty acids by pigs. Fatty acids: ingested, white; deposited in tissues, black. *L*, lauric (and lower saturated) acids; *M*, myristic acid; *P*, palmitic acid; *S*, stearic acid; *H*, hexadecenoic acid; *O*, oleic acid; *Ln*, linoleic acid; *A*, unsaturated C_{20-22} acids.

in the tissues of the four experimental animals fattened to 200 lb. live weight and of the two animals killed at the age of 16 weeks. Fig. 1 is a graphical presentation of the same data.

Table VII. *Comparison of acids in fat ingested and deposited by pigs*

(*a*) Pigs at different planes of nutrition to 200 lb. wt.

Pig	73 (Hog)			74 (Gilt)		
Planes of nutrition	Low-High			High-High		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C_{14}	0·11	Trace	−0·11	0·11	—	−0·11
Myristic	0·24	0·29	+0·05	0·26	0·24	−0·02
Palmitic	1·28	8·27	+6·99	1·53	6·92	+5·39
Stearic	0·25	3·99	+3·74	0·24	3·58	+3·34
Unsaturated:						
C_{16} (and C_{14})	0·22	0·89	+0·67	0·22	0·72	+0·50
Oleic	3·24	13·87	+10·63	3·80	10·50	+6·70
Linoleic	3·30	1·48	−1·82	3·22	1·43	−1·79
C_{20-22}	0·87	0·74	−0·13	0·86	0·48	−0·38
	9·51	29·53	+20·02	10·24	23·87	+13·63

Pig	72 (Hog)			82 (Gilt)		
Planes of nutrition	High-Low			Low-Low		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C_{14}	0·12	Trace	−0·12	0·16	Trace	−0·16
Myristic	0·24	0·21	−0·03	0·27	0·15	−0·12
Palmitic	1·36	5·45	+4·09	1·28	4·00	+2·72
Stearic	0·20	2·99	+2·79	0·25	2·16	+1·91
Unsaturated:						
C_{16} (and C_{14})	0·17	0·43	+0·26	0·22	0·31	+0·09
Oleic	3·27	10·69	+7·42	3·07	7·09	+4·02
Linoleic	2·44	1·07	−1·37	2·85	1·23	−1·62
C_{20-22}	0·66	0·53	−0·13	0·74	0·46	−0·28
	8·46	21·37	+12·91	8·84	15·40	+6·56

(*b*) Pigs at different planes of nutrition to 16 weeks

Pig	138 (Gilt)			139 (Gilt)		
Planes of nutrition	High			Low		
Fatty acids (kg.)	Fed	Deposited	Difference	Fed	Deposited	Difference
Saturated:						
Below C_{14}	0·08	Trace	−0·08	0·02	—	−0·02
Myristic	0·14	0·13	−0·01	0·04	0·01	−0·03
Palmitic	0·87	3·81	+2·94	0·32	0·15	−0·17
Stearic	0·07	1·14	+1·07	0·01	0·06	+0·05
Unsaturated:						
C_{16} (and C_{14})	0·07	0·51	+0·44	0·01	0·03	+0·02
Oleic	1·97	6·48	+4·51	0·75	0·23	−0·52
Linoleic	0·73	0·38	−0·35	0·08	0·04	−0·04
C_{20-22}	0·21	0·25	+0·04	0·02	0·01	−0·01
	4·14	12·70	+8·56	1·25	0·53	−0·72

It is an interesting point that the only animal which did not deposit more fat than it ingested is the 16-weeks old pig fed on a restricted ration. At the same time, it will be seen that, even at this very low plane of nutrition, obvious fat synthesis took place during the subsequent stages of growth. The fat deposition in the four 200 lb. weight pigs and in the 16-weeks old pig fed on the high plane of nutrition is not only well marked, but follows the same lines in each case as regards the individual fatty acids which have been laid down.

The amounts of palmitic, oleic and stearic acids in the deposited fats greatly exceed those ingested in the respective diets, and show that the fat synthesized by the animal from non-fatty sources consists predominantly of glycerides of these three acids. The same conclusion applies to the hexadecenoic acid in the depot fats which, though relatively small in amount, is usually several times as much as can possibly have been present in the dietary fat. Myristic acid, however, is present in about the same amount in the animal fats as in the ingested fats, and the evidence as to its origin is therefore inconclusive; unless the absorption of myristic glycerides from the diet was extremely complete, some of this acid must also have been synthesized.

The remaining pig fat component acids—linoleic acid, the unsaturated C_{20-22} acids and saturated acids below myristic—show an opposite relationship to the respective ingested fatty acids: the amount deposited is consistently less than that present in the diet. It will be noticed that, both in this group and in the preceding group (where evidence of fat synthesis is pronounced), the respective differences for each of the animals show, on the whole, a remarkable and often almost quantitative degree of similarity.

It is clear that fats containing saturated acids of lower molecular weight than myristic acid are neither synthesized nor assimilated by the pig; this accords with the observations of other workers on the body fats of rats and some other animals.

The quantity of linoleic acid in the body fats is not more than (and usually definitely less than) half of that available in the dietary fats; this suggests strongly, although it does not afford positive proof, that, like the rat, the pig is not able to synthesize linoleic acid and derives glycerides of this acid only by assimilation. This view may receive further support from the fact that the octadecadienoic acid of pig depot fats, unlike that of ox depot fat [Hilditch & Longenecker, 1937] has the properties of seed-fat linoleic acid [Hilditch & Stainsby, 1935]. Ellis & Zeller [1930] similarly concluded that, in the pig, linoleic acid is not synthesized, but comes from the food, except possibly in the case of young animals.

The quantity of unsaturated C_{20-22} acids present as glycerides in the depots likewise falls short of that present in the diet (in this instance in the fish meal constituents); but the disparity is less pronounced than in the case of linoleic acid, and the possibility of some slight degree of synthesis of the acids of this group by the pig (especially in the first weeks of its life) cannot be excluded. For the most part, however, they seem to be derived from the fish meal present in the diet. It was pointed out earlier that the highly unsaturated C_{20-22} acids in the pig differ in properties from the corresponding acids of the fish meal, and selective absorption of some of the group is perhaps an alternative possibility.

Whilst it cannot, of course, be taken for granted that the increases recorded in Table VII in palmitic, stearic and oleic acids represent the total amounts of each of these acids which have been synthesized, it is of interest to note that the ratios of the increases of palmitic to those of the two C_{18} acids are as follows:

Pig no.	Palmitic	Stearic + oleic	Palmitic : C_{18} acids
73	6·99	14·37	1 : 2·06
74	5·39	10·04	1 : 1·86
72	4·09	10·21	1 : 2·50
82	2·72	5·93	1 : 2·18
138	2·94	5·58	1 : 1·90

The mean ratio for the whole series is 1 : 2·08 (wt.) or 1 : 1·89 (mol.). Palmito-di-C_{18} glycerides (especially palmitodiolein and palmito-oleostearin) have

previously been shown to be the main components of pig depot fats [Hilditch & Stainsby, 1935], and the present figures point to these as the predominant glycerides in the fat produced by synthesis in this animal.

Summary

The component acids of a series of depot (back) fats from pigs fed on different planes of nutrition have been determined. On a restricted diet the deposition of fat is not only slower, but the fat produced is softer, owing to increase in the small proportions of linoleic acid present, together with some increase in the proportion of oleic acid.

Comparison of the amounts of each component acid in the total fats present in the diets with those in the body fats of the animals shows substantial synthesis of glycerides of palmitic, oleic and stearic acids, in the average proportion of 1 mol. palmitic to 1·9 mol. C_{18} acids. The minor components hexadecenoic and (possibly) myristic acid may also be mainly formed by synthesis, but linoleic and unsaturated C_{20-22} acids in the depots are derived only from ingested fats.

Our cordial thanks are offered to Dr J. Hammond for his courtesy in providing the experimental material and allowing us to consult his data as regards the diet and the composition of the tissues of the animals, and to Dr E. H. Callow for assistance in the extraction and the determination of the general characteristics of the fats in the feeding-stuffs and in some of the pig depots. The work described in this communication forms part of a research programme undertaken by the Food Investigation Board of the Department of Scientific and Industrial Research, to whom we are indebted for permission to publish the results.

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LXI. THE DIETARY PREVENTION OF FATTY LIVERS. COMPOUNDS RELATED TO CHOLINE

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THE relative activities of various compounds related to choline in preventing the deposition of fat in the livers of rats maintained on a diet high in fat and low in other lipotropic factors have been studied as a possible source of information as to the mode of action of choline itself [Channon & Smith, 1936; Channon *et al.* 1937; Welch, 1936; Welch & Welch, 1938]. The present paper records similar investigations of a number of other compounds.

Two of the compounds used, cholamine and betaine, had been studied previously by Best & Huntsman [1932]. While cholamine had been found to be inactive, betaine had prevented the deposition of liver fat, a result confirmed by Welch & Welch [1938]. The experiments of Best & Huntsman, however, were carried out with the 40% fat-60% grain diet and it seemed desirable to repeat the work using a "synthetic" choline-free diet; moreover, the latter authors provided no evidence regarding the relative activity of betaine in terms of choline. Welch & Welch [1938] have since suggested that it has rather more than half the activity of choline. In view of the fact that liver tissues can oxidize choline to betaine aldehyde and probably to betaine [Mann & Quastel, 1937; Bernheim & Bernheim, 1938; Mann *et al.* 1938], the degree of activity of betaine is a point of some importance since it might possibly be the compound ultimately active when choline supplements prevent liver fat deposition.

Another compound tested was creatine, since several authors have suggested that choline is a precursor of creatine in metabolism [Riesser, 1913; 1914; Baumann & Hines, 1918; Shanks, 1921; Klee & Petropuliades, 1928]. Finally, two further compounds related to choline were investigated: choline methyl ether and tetra(β-hydroxyethyl)ammonium chloride. Since choline might act by enabling lecithin formation to take place it was decided to study the methyl ether of choline, to see whether etherification of the alcohol group robbed the compound of its lipotropic activity. The results with the tetrahydroxy derivative should show whether the alkyl groups were necessary for the activity found in choline and some of its analogues.

EXPERIMENTAL

Choline methyl ether was prepared by a method based on that described by Renshaw [1910] for choline. β-Chloroethyl methyl ether, prepared from the monomethyl ether of ethylene glycol by the action of phosphorus pentachloride, was sealed with a molecular proportion of trimethylamine in a bomb tube and heated at 95° for 6 hr. with occasional shaking. The contents of the tube were then dissolved in alcohol, purified by treatment with charcoal and precipitated by means of acetone and ether. The crystallized material was purified by similar means. Exact analysis was rendered difficult by its extremely hygroscopic

nature but the chloroaurate was a stable compound and had M.P. 155°. (Found: C, 15·90; H, 3·59; Au, 43·38%. $C_6H_{16}ONAuCl_4$ requires C, 15·75; H, 3·51; Au, 43·15%.)

The tetra(β-hydroxyethyl)ammonium chloride used in these experiments was a sample kindly given to us by Mr A. L. Bacharach.

Animal feeding and liver analyses

Groups of 10 rats were fed on a basal diet producing a fatty liver, one group serving as a control and the others receiving supplements of the various compounds or of choline itself, the supplements in aqueous solution being mixed in the diet. The choline content of the basal ration was negligible except in exp. 5 when marmite was included to supply vitamin B complex, and provided about 1·5 mg. choline per rat per day, the effect of which, it is considered, would be negligible. Except in exp. 5, the individual livers were analysed by digesting with 40% NaOH and refluxing with alcohol, the fatty acids and unsaponifiable fraction then being extracted with ether and subsequently purified by treatment with light petroleum: in these cases "fat" represents fatty acids plus unsaponifiable matter. In exp. 5 the pooled livers were ground with sand and extracted with alcohol and ether and in this case therefore "fat" represents the total ether-soluble material so obtained. Details of the diets differed in the various experiments and are considered when discussing the results.

Results

Betaine. The basal diet used in these experiments consisted of beef dripping 40, protein 8, glucose 46, salt mixture 5, cod-liver oil 1 part. In addition aneurin was added to the diet so that each animal received about 10 μg. daily. The protein fraction was egg albumin in exps. 1 and 3 and caseinogen (alcohol-ether-extracted) in exp. 2. In exps. 1 and 3 the animals were all bucks while in exp. 2 there were 5 bucks and 5 does in each group. Betaine hydrochloride and choline chloride were used but the amounts are expressed in terms of the free bases.

The first two experiments were, of necessity, preliminary in nature, since none of the figures recorded in the literature was of any assistance in forming an estimate as to the lipotropic activity of betaine relative to that of choline. All the relevant data are presented in Table I together with those of exp. 3.

On the animal side, both exps. 1 and 2 are obviously satisfactory, the weight losses not being such as to interfere with the interpretation of the results.

In the first experiment 0·1% choline and betaine were given to two groups respectively and it can be seen that while the former supplement has prevented a large increase in the amount of fat deposited in the liver, the presence of 0·1% betaine has not had a very marked effect. The livers of the control animals contained 27·01% fat, and those of the choline group 8·18%, values to be contrasted with the 22·14% present in the livers of the animals receiving betaine. In our experience, the difference between the control and betaine groups is hardly significant. The figures for the amount of fat in the liver of the 100 g. rat, 1·82 and 1·28 g., show, however, a larger difference and it was accordingly decided to increase the betaine and to reduce the choline dosage in the next experiment.

Before considering the results obtained in exp. 2, it must be pointed out that the wide difference between the two control values for liver fat obtained in exps. 1 and 2 is due to the fact that in the first, egg albumin was the basal protein, while in the second it was caseinogen; this point has already been discussed by Channon *et al.* [1938]. From the results of exp. 2, it can be seen that

Table I. *Effect of betaine supplements in preventing liver fat deposition*

Group	No. of rats	Change in wt. ± % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.	Base intake mg./rat/day	Choline equivalent mg./rat/day
Exp. 1. Period of feeding 18 days:							
1. Control	8	+4·8	6·6	27·01	1·82	Not recorded	
2. +0·1% choline	10	+5·0	5·2	8·18	0·41		
3. +0·1% betaine	10	+8·6	5·4	22·14	1·28		
Exp. 2. Period of feeding 17 days:							
4. Control	9	−9·1	3·9	17·03	0·70	Not recorded	
5. +0·03% choline	10	−6·3	3·9	15·51	0·65		
6. +0·05% choline	10	−5·1	3·6	9·58	0·36		
7. +0·20% betaine	10	−7·9	3·3	8·50	0·28		
8. +0·50% betaine	10	−5·1	3·5	4·80	0·17		
Exp. 3. Period of feeding 18 days:							
9. Control	10	+1·3	4·8	24·10	1·24	—	—
10. +0·03% choline	10	0	4·1	15·70	0·71	2·8	2·8
11. +0·05% choline	10	+1·0	3·8	13·18	0·51	4·4	4·4
12. +0·07% choline	10	−2·2	3·7	8·79	0·32	6·5	6·5
13. +0·10% betaine	10	+1·8	4·1	16·49	0·76	9·4	8·4
14. +0·15% betaine	10	+2·2	4·1	15·23	0·68	14·1	12·7
15. +0·20% betaine	10	−1·0	3·7	12·41	0·48	17·2	15·4

the groups 6, receiving 0·05% choline, and 7, receiving 0·20% betaine, have given practically the same values for liver fat, 9·58 and 8·50%, the control figure being 17·03%; the absolute amounts support this conclusion. It is to be noted that in this experiment the inclusion of 0·50% betaine has maintained the liver fat at nearly the normal level, 4·80% and 0·17 g. in the 100 g. rat. This may be of significance in view of the fact that on several occasions even massive doses of choline have failed to show so marked an action in preventing fatty infiltration.

These results suggested that betaine had a little more activity than 25% of its weight of choline in preventing fat deposition in the liver, a conclusion which may be better expressed by saying that it is slightly more active than 32% of an equimolar amount of choline. Exp. 3 was designed to test this finding and seven groups of animals were used, six of them receiving graded quantities of choline and betaine as set out in Table I. The food intakes of the various groups were measured in order to show exactly the intake of base in each group.

In exp. 3 the weight changes and food intakes (8·6–9·4 g./rat/day) are comparable in all the groups. The figures for both the percentage of fat in the liver and the weight in the liver of the 100 g. rat show that 4·4 mg. choline and 17·2 mg. betaine have had substantially the same preventive action, the results being 13·18% and 0·51 g., 12·41% and 0·48 g. respectively, as compared with the control values of 24·10% and 1·24 g. In Table I the amounts of betaine administered are converted into their equivalents in terms of choline, and comparing the choline and betaine intakes in this form the results of groups 11 and 15 indicate a 29% activity for betaine. There is no significant difference between the groups which received 9·4 mg. and 14·1 mg. betaine, a fact which can only be referred to the individual variations among the animals. However, the values obtained for liver fat, 16·49 and 15·23% respectively, are similar to that obtained in the case of 2·8 mg. choline, 15·70%, while 100 g. rat figures for the three groups are 0·76, 0·68 and 0·71 g. respectively. The activity of betaine in these cases is 33 and 22% of that of choline. From the approximate value of 32% suggested by the results of exp. 2, and the values 29, 33 and 22% obtained

in this more accurate experiment, it is reasonable to conclude that betaine has about 30 % of the efficiency of choline in preventing fatty infiltration into the liver under these conditions.

Cholamine and creatine. The groups of animals used in this experiment consisted entirely of bucks and the same control group served for this experiment as for exp. 1. Two groups received, in addition to the basal 8 % egg albumin diet, 1 % cholamine and 0·5 % creatine respectively.

Table II. *The effect of cholamine and creatine in fatty liver prevention*

Group	No. of rats	Change in wt. ± % initial body wt.	Liver % final body wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 4. Period of feeding 18 days:					
1. Control	8	+4·8	6·6	27·01	1·82
16. +1 % cholamine	9	+4·1	6·0	25·64	1·67
17. +0·5 % creatine	10	+1·1	6·4	27·96	1·71

Clearly neither compound has exerted any lipotropic action, for the groups of animals receiving them have livers as fatty as those of the control group, and in view of the relatively large quantities administered, it was not considered profitable to pursue the investigations any further.

Choline methyl ether and tetra(β-hydroxyethyl)ammonium chloride. In view of the considerable toxicity which the choline analogues previously investigated had been found to possess [Channon & Smith, 1936; Channon, Platt & Smith, 1937], it was decided to use relatively small quantities of these two compounds in preliminary experiments. The tetrahydroxyethyl derivative was tested first and two groups of does received a basal diet calculated to produce the "cholesterol" fatty liver: caseinogen (alcohol-ether-extracted) 5, beef dripping 30, glucose 52, marmite 5, salt mixture 5, cholesterol 2, cod liver oil 1 part. One group served as control and the other received a supplement of 0·14 % of the tetrahydroxyethyl derivative. The experimental data for this and the subsequent exps. 6, 7 and 8 are presented in Table III.

Table III. *Effect of choline methyl ether and tetra(β-hydroxyethyl)ammonium chloride in fatty liver prevention*

Group	No. of rats	Change in wt. ± % initial body wt.	Liver % final wt.	Fat % liver wt.	Fat in liver of 100 g. rat g.
Exp. 5. Period of feeding 14 days:					
18. Control	10	−2·3	5·5	24·3	1·34
19. +0·14 % tetrahydroxyethyl derivative	9	+1·0	5·2	29·4	1·52
Exp. 6. Period of feeding 12 days:					
20. Control	10	−11·3	4·1	16·36	0·68
21. +0·10 % choline	10	− 9·6	3·5	7·74	0·30
22. +0·11 % choline methyl ether	9	−13·6	4·1	17·09	0·74
23. +0·25 % tetrahydroxyethyl derivative	10	−10·7	4·3	15·76	0·67
Exp. 7. Period of feeding 18 days:					
1. Control	8	+ 4·8	6·6	27·01	1·82
2. +0·10 % choline	10	+ 5·0	5·2	8·18	0·41
24. +0·28 % choline methyl ether	10	+ 1·7	5·6	24·46	1·41
Exp. 8. Period of feeding 17 days:					
4. Control	9	− 9·1	3·9	17·03	0·70
6. +0·05 % choline	10	− 5·1	3·6	9·58	0·36
25. +0·50 % choline methyl ether	9	−14·3	3·6	16·32	0·62

The figures for weight change show that the compound is in no way toxic, while from the analytical figures it appears to have had little action on liver fat deposition. Actually the percentage of fat in the livers of the group receiving the tetrahydroxyethyl derivative, 29·4, is higher than in the control group, 24·3, but the difference in the absolute weights, which are 1·52 and 1·34 g., is not so great. While these groups do not differ significantly, further evidence as to the effect of a larger dose of the tetrahydroxyethyl derivative was clearly desirable, and accordingly this compound was again fed in exp. 6 in which choline methyl ether was also studied.

Four groups of does were maintained on a basal diet consisting of caseinogen (alcohol-ether-extracted) 5 parts, beef dripping 40, glucose 49, salts 5, cod liver oil 1 part, each animal receiving in addition 10 μg. aneurin daily. Two groups received 0·11 % choline methyl ether and 0·25 % tetra(β-hydroxyethyl)-ammonium chloride respectively, while a third group received 0·10 % choline to facilitate the assessment of lipotropic action.

The weight losses in exp. 6, although rather large, are not such as to interfere with any conclusions which may be drawn, and from the point of view of weight maintenance, the superiority of egg albumin as a basal protein in these low-protein diets is clearly demonstrated (compare groups 1 and 9 with 4 and 20). From study of these results it is seen that the tetrahydroxyethyl derivative has no choline-like action on liver fat, nor has the methyl ether in the amount employed. The percentages of liver fat in these two groups, 15·76 and 17·09 respectively, are substantially the same as the control group's 16·36 %, a conclusion borne out by the values obtained for the weights in the 100 g. rat, 0·68, 0·74 and 0·67 g., for the control, choline methyl ether and tetrahydroxyethyl compound groups respectively. In contrast with these, group 21, which received 0·10 % choline, had only 7·74 % liver fat or 0·30 g. in the 100 g. rat, figures which demonstrate very clearly the powerful action of choline in preventing the deposition of liver fat under these conditions.

Clearly tetra(β-hydroxyethyl)ammonium chloride had no lipotropic action but the lack of toxicity of the choline methyl ether made it desirable to study its action in large dosages, and two further experiments were therefore carried out using this derivative with the results quoted in Table III. In exp. 7 the animals used were bucks and the basal diet consisted of egg albumin 8, beef dripping 40, glucose 46, salt mixture 5, cod liver oil 1 part and 10 μg. aneurin per rat per day. The same diet was used in exp. 8 with the substitution of caseinogen for egg albumin as the protein while in this case the animals consisted of equal numbers of both sexes.

Considering the results of exp. 7 first, it would appear just possible that the choline ether possesses some activity, for although the results obtained for this group, 24·46 % and 1·41 g., are not markedly different from the control values, 27·01 % and 1·82 g., nevertheless they are somewhat lower. In view of the marked effect of 0·1 % choline, which has prevented an increase in fat content of nearly 19 % of the wet liver weight, any action of the ether must be slight and it was therefore decided to use a massive dosage, 0·5 %, in the next experiment.

In exp. 8, the choline methyl ether has clearly exercised no lipotropic action whatsoever, the values both for the percentage of liver fat and for the weight in the 100 g. rat not differing significantly from those of the control group. On the other hand, only 0·05 % choline has had a marked preventive action. The negative result obtained with this large dose provides confirmation of the conclusion from exps. 6 and 7 that choline methyl ether has no activity in preventing this fatty infiltration into the liver.

Discussion

In the present work betaine has been found to possess about 30% of the activity of choline in preventing fatty infiltration in the livers of rats maintained on choline-free diets of high fat and low protein contents. This finding that betaine is lipotropically active confirms previous studies by Best & Huntsman [1932] and by Welch & Welch [1938], the latter authors also finding betaine aldehyde to be active. Neither paper, however, offers evidence as to the relative efficiencies of the two compounds although Welch & Welch [1938] suggest that betaine may have more than 50% of the activity of choline, but defer a definite statement until a later date. Quastel and his associates [1937; 1938] and Bernheim & Bernheim [1938] having shown that choline is oxidized to betaine aldehyde and then probably to betaine by surviving rat liver tissue and by enzyme systems extracted from rat liver, the present finding that betaine is less active than choline in fatty liver prevention suggests that, among other possibilities, it may be first converted into choline in the liver, this choline then being available for use: if this should be so, it would provide an interesting example of a readily reversible reaction taking place in animal tissues. Another explanation may be that while betaine itself is the active material, its activity is fully manifested only when it is produced in the liver by the oxidation of choline *in situ*, a part only of any dietary betaine being available and the rest oxidized before it is able to exercise its lipotropic action.

The very definitely negative result obtained with cholamine in exp. 4 confirms the work of Best & Huntsman [1932] and shows that if, in fatty liver prevention, lecithin formation resulting from choline feeding is the means whereby choline acts, the analogous synthesis of kephalin under similar conditions either plays no part, or cannot be influenced by cholamine feeding. This conclusion may indicate that kephalins have little or no importance in the metabolism of dietary fatty acids.

The results of the experiment with creatine indicate that this compound cannot serve as a source of choline in the body.

The results obtained with choline methyl ether show that if the primary alcohol group is etherified, choline becomes inactive. Apparently the body cannot remove a methyl ether group of this type although it appears to be able to demethylate methionine quite readily. Here again no light is thrown on the way in which choline may act, because, while the inactivation may be due to the fact that the choline ether can no longer be linked up in a phosphatide molecule, etherification may easily have inactivated the compound, whatever its mode of action.

Finally, study of tetra(β-hydroxyethyl)ammonium chloride has revealed the necessity that the alkyl radicles of the choline molecule be intact for the possession of lipotropic properties, a finding in agreement with the complete lack of activity of cholamine.

Summary

1. Studies have been made of five compounds—betaine, cholamine, creatine, choline methyl ether and tetra(β-hydroxyethyl)ammonium chloride—in order to discover whether they possess choline-like properties in preventing the deposition of liver fat under certain dietary conditions.

2. Of these compounds, only betaine was found to possess lipotropic properties. Its activity in fatty liver prevention was estimated to be approximately 30% of that of choline.

3. The relationship of these results to the theory that choline acts by enabling lecithin formation to take place is discussed.

I wish to record my indebtedness to Prof. H. J. Channon for helpful criticism and advice; and to Mr A. L. Bacharach for the gift of the tetra(β-hydroxyethyl)-ammonium chloride. The expenses of this research were defrayed in part by a grant to Prof. H. J. Channon from the Medical Research Council, to whom I wish to make grateful acknowledgement.

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LXII. THE INFLUENCE OF PHYTIN AND OF FATS ON THE PRODUCTION OF RICKETS BY A CEREAL DIET

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In an earlier paper [1937] we showed that the tendency of cereals to produce rickets entirely disappeared when calcium lactate was added so as to make the Ca/P ratio 1·0 : 0·5.

Other explanations for the rachitogenic action of cereals have been put forward. Bruce & Callow [1934] considered that the phosphorus (approximately one-half) which is combined as phytin in cereals is not available. McCance & Widdowson [1935] estimated the amount of phytin in 64 foodstuffs and by means of feeding experiments on four human subjects, using phytin obtained from Messrs Ciba Ltd., estimated that 20–60 % of the phytin ingested was excreted unchanged in the faeces.

In the present series of experiments the authors have used similar phytin from Messrs Ciba Ltd., adding it in varying proportions to a diet consisting otherwise entirely of cereals; these diets have then been fed to white Wistar rats. At the same time for comparison other rats were fed on the same diet without added phytin but with varying amounts of calcium lactate added to it.

The experiments consisted of feeding these diets to the rats for 5 weeks, noting the increase in weight and then, after killing the rats, estimating the amount of rickets by examination of the bones stained with silver nitrate. Projection drawings of the stained bones were made and four categories were recognized, (1) severe rickets (+ + +), (2) rickets (+ +), (3) slight rickets (+), and (4) normal bones (0).

Additional information was obtained by determining the amount of bone ash in the dry, fat-extracted bones. By this means it was thought that any tendency of the phytin-P to produce rickets or prevent the proper formation of bones would be disclosed.

A summary of the results of these experiments is given in Table I.

A consideration of these results shows at once that the added phytin has no prohibitive action on bone formation. On the contrary the addition of 1·5 % greatly reduces the rickets and 5 % completely prevents it. With a gradual increase of the amount added, and consequent reduction of the disproportion of P to Ca in the diet, we have a gradual improvement in the character of the bone and a rise in the ash content.

Comparison with the results obtained by the addition of calcium lactate is best made by first considering the Ca/P ratios and then the percentage of each substance added. Thus, for equal additions of 0·75 % of calcium lactate and phytin (nos. 2 and 3), the ratio in one case is 1 : 2·5 and in the other 1 : 3·5 and we have slight and severe rickets and bone ash percentages of 40·5 and 34·6. If, however, we consider the ratio 1 : 2·5 we find that with the addition of 0·75 %

Table I

Diet	Av. initial wt. of 3 rats g.	Av. gain of 3 rats g.	Bone condition at end of test	Bone ash %	Ca : P ratio calculated	Ref. no.
*Germ flour 100%	50	17	+ + +	28·3	1 : 9	1
			+ + +			
			+ + +			
+0·75% Ca lactate	48	57	+	40·5	1 : 2·5	2
			+			
			+			
+0·75% phytin	48	17	+ + +	34·6	1 : 3·5	3
			+ +			
			+ + +			
+1·5% Ca lactate	48	64	0	50·68	1 : 1·4	4
			0			
			0			
+1·5% phytin	48	45	+	40·4	1 : 3·0	5
			0			
			0			
+3·0% Ca lactate	55	90	0	60·7	1 : 0·45	6
			0			
			0			
+3·0% phytin	48	51	+	43·0	1 : 2·5	7
			0			
			0			
+5·0% phytin	58	55	0	52·8	1 : 2·0	8
			0			
			0			
+3·0% Ca lactate +5% phytin	55	71	0	56·3	1 : 1·3	9
			0			
			0			

* The germ flour used consisted of a mixture of 75% white flour and 25% sterilized wheat germ.

calcium lactate we get slight rickets and bone ash of 40·5% and by the addition of 3% phytin to produce a similar ratio we get slight rickets and bone ash of 43·0% (No. 7). These amounts of calcium lactate and phytin represent in the case of lactate an addition of 0·1 g. of Ca and in the case of phytin an addition of 0·36 g. Ca. This actually tends to confirm our previous contention that it was the disproportion of P to Ca rather than the presence of any other substance which caused the rickets, especially if one considers experiment No. 9 where an addition of 3% calcium lactate and also 5% phytin was made. Here the additions of lactate and phytin made are equivalent to 1·0 g. Ca and 1·05 g. P to a diet containing 0·04 g. Ca and 0·36 g. P, resulting in a Ca/P ratio of 1 : 1·3. The results obtained were good but not equal to those of experiment No. 6 where only lactate was added, equivalent to an addition of 0·4 g. Ca and no P and resulting in a Ca/P ratio of 1 : 0·43, which is much closer to the optimum ratio of 1 : 0·5.

If, as is suggested by McCance & Widdowson [1935], 20–60% of the P in phytin is unavailable, the Ca/P ratios for experiments Nos. 7 and 9 might be assumed to be 1 : 1·5 and 1 : 0·7. The results, however, indicate that a much higher percentage of the phytin-P is utilized.

A recent paper by McDougall [1938] suggests that the additions of fats, olive oil or lard, prevents the occurrence of rickets on a cereal diet by creating favourable conditions for the absorption of Ca. It is inferred that an insoluble calcium phosphate is precipitated in the intestine by the action on phytin of an enzyme

which splits it into soluble sodium phosphate which then reacts with Ca to form insoluble calcium phosphate.

The above results did not confirm this suggestion and further experiments were started to see whether it would be possible to obtain results similar to McDougall's by the addition of fat to the diet. In these experiments mixtures of germ flour, casein and varying proportions of calcium lactate were made. These were fed to rats with the addition in the experiments indicated of 10% of vitamin-free hardened vegetable oil. Vitamin-free oil was chosen in order to remove any possibility of the results being affected solely by vitamin D and not by the fatty acids of the oil.

After 5 weeks the rats were killed and the bones examined for rickets as before. Table II shows the results obtained.

Table II

Diet (%)		Initial wt. of rat g.	Gain in wt. g.	Bone condition at end of test	Bone ash %	Ca : P ratio calculated	Ref. no.
Germ flour	73·5	50	100	0	Not	1 : 1·2	1
Casein	24·5	50	95	0	available		
Ca lactate	2·0	45	105	0			
Germ flour	66·0	50	90	0	Not	1 : 1·5	2
Casein	22·0	50	90	0	available		
Ca lactate	2·0	55	80	0			
Hard. veg. oil	10·0						
Germ flour	73·7	50	90	0	54·6	1 : 1·3	3
Casein	24·5	50	60	0			
Ca lactate	1·75	55	80	+			
Germ flour	66·15	50	90	+ +	53·1	1 : 1·4	4
Casein	22·10	50	60	+			
Ca lactate	1·75	Found dead					
Hard. veg. oil	10·0						
Germ flour	74·25	50	90	+ +	46·5	1 : 1·4	5
Casein	24·25	50	50	0			
Ca lactate	1·5	55	125	+ + +			
Germ flour	66·5	55	80	+ +	47·5	1 : 1·5	6
Casein	22·0	45	50	+ +			
Ca lactate	1·5	45	55	+			
Hard. veg. oil	10·0						
Germ flour	74·5	60	70	+	52·3	1 : 1·8	7
Casein	24·5	55	65	+			
Ca lactate	1·0	60	50	+			
Germ flour	74·5	50	55	+ + +	47·2	1 : 2·5	8
Casein	25·0	50	60	+ + +			
Ca lactate	0·5	50	45	+ +			
Germ flour	67·0	50	50	+ +	47·5	1 : 2·5	9
Casein	22·5	50	50	+ + +			
Ca lactate	0·5	50	60	+			
Hard. veg. oil	10·0						
Germ flour	75·0	45	50	+	37·3	1 : 3·2	10
Casein	25·0	45	60	+			
		50	40	+			
Germ flour	67·5	50	40	+ + +	29·1	1 : 3·5	11
Casein	22·5	45	40	+ + +			
Hard. veg. oil	10·0	50	45	+			

These results show no decided prevention of rickets by the addition of oil, yet such experiments in which a diet is given to produce not severe rickets but mild or moderate rickets, ought easily to show the preventive action of oil. The

experiments show that, when such diets are given, to obtain consistently normal bones not less than 2 % of calcium lactate must be added representing a calculated Ca/P ratio of 1 : 1·2.

SUMMARY

1. Our previous finding is confirmed that the tendency of cereals to produce rickets resides in their low Ca and high P contents, and that this tendency can be entirely counteracted by adding calcium lactate so as to make the Ca/P ratio 1 : 0·5.

2. The rachitogenic action of cereals does not reside in the phytin which they contain, for the reason that the addition of phytin instead of increasing this action reduces the rickets.

3. The addition of a vegetable oil free from vitamin D does not prevent the occurrence of rickets resulting from a cereal diet; here we fail to confirm the findings of McDougall.

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LXIII. SOME PROPERTIES OF THE REDUCING MATERIAL IN CERTAIN FRACTIONS OF NORMAL URINES

III. SOME OBSERVATIONS ON THE NATURE OF THE NON-FERMENTABLE REDUCING SUBSTANCES IN "FASTING" URINE

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In the two preceding papers of this series [Harding *et al.* 1936, 1, 2] the nature of the "free fermentable sugar" in urine and of the substances which yield fermentable sugar on hydrolysis was discussed. No attempt was made at that time to determine the nature of the reducing substances which were non-fermentable even after hydrolysis. Everett & Sheppard [1930; 1931] and Everett *et al.* [1934, 1] presented evidence indicating the presence of a substance which they named uroketose. It is precipitated by neither Hg^{++} nor Ba salts in neutral solutions. It is resistant to bromination at room temperature and gives rise to 2-furfuraldehyde when heated with acid. Everett claims that this substance, which differs from ketoxylose, constitutes the greater part of the reducing substance of normal urine.

It was thought that further information as to the nature of the residual reducing substances in normal human urine might be gained by the application of the modification of Everett *et al.* [1934, 1] of the Votocek and Nemecek technique of Br oxidation and a modified NaOI oxidation method to urine fractions obtained by the copper-lime method [Archibald, 1935; Harding *et al.* 1936, 1] which gives solutions of the urine sugars free from nitrogen and phenols. These procedures were therefore first tested on dilute sugar solutions of about the same concentration as the reducing substances found in the urine fractions and then applied to the urine fractions.

Analytical methods

Composite fasting urines were collected, diluted and treated with Pb acetate [Harding *et al.* 1936, 1, 2], $HgSO_4$ [West *et al.* 1929] and copper-lime [Harding *et al.* 1936, 1, 2].[2] Further fractionation was accomplished by acid hydrolysis and NaOI oxidation according to the steps illustrated in the diagram.

[1] Part of the data embodied in this paper was taken from a thesis submitted by one of the writers (R. M. A.) in partial fulfilment of the requirements for the degree of Ph.D. in the University of Toronto.

[2] In view of the fact that urine may contain considerable amounts of diastase, starch-free filter papers were used throughout these experiments as well as in those previously reported.

Six 4 hr. fasting normal urines—Dilution 1·14
Basic Pb acetate—Dilution 5
$HgSO_4$-$BaCO_3$—Dilution 10
Evaporate under reduced pressure
Fraction A—Dilution 3
Cu-lime (at dilution 0·380)

Cu-lime precipitate *Fraction B*—Dilution 2·5		Cu-lime filtrate *Fraction G*—Dilution 1·0	
Hydrolysis—*Fraction C*—Dilution 2·74		Hydrolysis Cu-lime	Hypoiodite Hydrolysis Cu-lime
Hypoiodite—*Fraction F* Dilution 3·42			
Hypoiodite—*Fraction D*—Dilution 3·17		Precipitate	Precipitate
Hydrolysis—*Fraction E* Dilution 3·47		*Fraction H* Dilution 0·346	*Fraction J* Dilution 1·38

Dilutions reported are based on the assumption that 100 ml. of urine per 4 hr. corresponds to Dilution 1.

Hydrolyses were carried out by boiling in *N* H_2SO_4 under a reflux condenser for 1 hr. NaOI oxidations were done by the method described by Harding *et al.* [1936, 2] except that the final dilutions were varied and the small amount of KI left after $HgSO_4$ treatment was precipitated as AgI by the addition of a slight excess of powdered Ag_2SO_4, the excess Ag being removed by the addition of a slight excess of HCl. The copper-lime filtrates required increased amounts of I_2 solution and of NaOH.

All fractions were adjusted to *p*H 6·5. "Reducing values" were determined by the Harding & Downs [1933] reagent and are expressed as mg. glucose per single 4 hr. specimen. Br oxidation curves were determined on Fractions B, C, D, E and F by the method of Everett *et al.* [1934, 1], using the Harding & Downs reagent to determine changes in reducing value. The quantity of furfural-yielding material was determined in Fractions B, C, D and E by McCance's [1926] technique, separating the two layers by centrifuging for 30 sec. instead of by standing, and the free sugar by differential fermentation [Harding & Nicholson, 1933] in Fractions B, C, E, H and J.

Discussion of methods

Although most of the solutions used were obtained by regenerating the precipitates after a single treatment with copper-lime and therefore contained small amounts of nitrogenous material [Archibald, 1935], determinations done on N-free solutions obtained by reprecipitating the material [Archibald, 1935; Harding *et al.* 1936, 1] gave parallel results. It is therefore certain that none of the phenomena observed can be due to N-containing substances.

NaOI rapidly destroys all the "reducing power" of common aldohexoses and aldopentoses and the furfural-producing properties of the latter; it is therefore a more satisfactory method than Br oxidation for differentiating ketose from aldoses such as mannose, which are oxidized but slowly by the Br. Everett *et al.* [1934, 1] report that using Kolthoff's method of NaOI oxidation in urines, 30% of the uroketose and added fructose is destroyed. It has been found, however, that the reducing power of fructose towards the Harding-Downs reagent, both in water solution and when added to urine, is almost unchanged by the NaOI or Br procedures used here. NaOI oxidation also completely removes the "reducing values" of free glucuronic acid, glucuronic acid monobenzoate, and borneol glucuronate both before and after hydrolysis as well as after 48 hr. treatment with Br.

The estimation of the amounts of furfural-producing substances present was of necessity very inaccurate because of the great differences in furfural yield between even such closely related substances as pentoses, e.g. arabinose yields three times as much furfural as does xylose.

In 48 hr. Br neither hydrolyses nor oxidizes appreciable quantities of glucuronic acid monobenzoate but it does hydrolyse 40 % of borneol glucuronate and destroys the reducing properties of the material thus liberated. Repeated Br oxidations on sugars 100 times as dilute as those used by Everett *et al.* [1934, 1] gave curves identical with those obtained by these authors. The Br oxidations gave results which fitted very well into a smooth curve. Little difficulty was experienced in repeating the results with a given fraction if the conditions influencing the rate of oxidation were carefully controlled.

The nature of the non-fermentable reducing substances of "fasting" urine

Typical results for the changes in reducing value towards the Harding-Downs reagent induced by NaOI oxidation and for the fermentable sugar in the various urine fractions are given in Table I. Fig. 1 represents the Br oxidation

Table I. *The fermentable sugars and the reducing substance destroyed by NaOI in various urine fractions*

	B	C	Increase	E	Increase	H	J
Total reduction	26·8	32·3	5·5	9·7	2·8	6·1	4·3
Fermentable sugar (including galactose)	10·3	16·6	6·3	3·0	3·0	1·8	1·0
Reducing material destroyed by NaOI	19·9	28·8	8·9	—	—	—	—

All results are expressed as mg. glucose/4 hr.

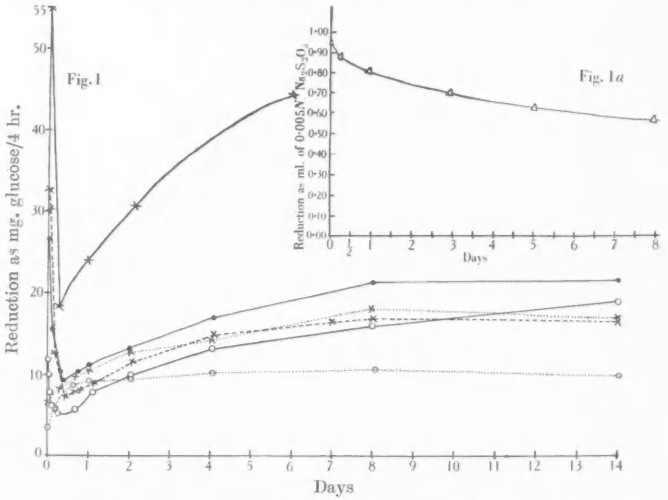

Fig. 1. The effect of Br oxidation on the reducing power of different urine fractions. Fraction B, •—•; Fraction C, ×- - - ×; Fraction D, ×· · · · ×; Fraction E, o—o; Fraction F, o· · · · o; $HgSO_4$-KOH filtrate, ×—×.

Fig. 1*a*. The effect of Br oxidation on the reducing power of glucuronic acid monobenzoate.

curves plotted over a period of 14 days and the curves obtained during the first 16 hr. of oxidation are recorded on a larger scale in Fig. 2. Although these are results obtained with male urines, composite specimens of female urine gave similar results.

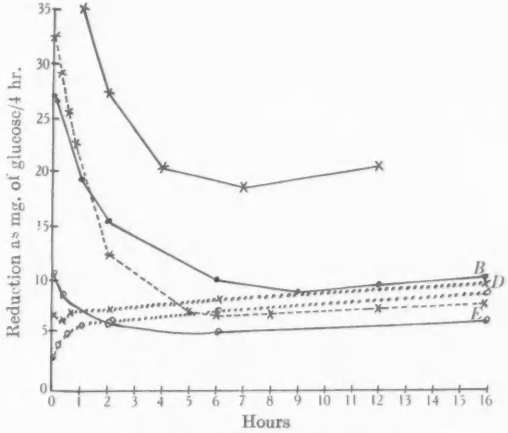

Fig. 2. For description see Fig. 1.

NaOI oxidation decreases the reducing values of Fraction B from 26·8 mg./4 hr. to 6·9 mg./4 hr. and of Fraction C from 32·3 mg./4 hr. to 3·5 mg./4 hr. Of the reducing material destroyed by NaOI in Fraction B, 50 % can be accounted for by the fermentable sugar present and of that destroyed in Fraction C, 57 %. This is a much greater proportionate drop in the reducing power than that observed by Everett *et al.* [1934, 1], but the difference between the two observations is to be expected when the much more drastic clearing procedure used here is considered, the copper-lime precipitation giving a fraction which is much more nearly a solution of carbohydrate only. Furfural-yielding materials represent a considerable portion of the aldose-like substance, the percentage depending on their reducing power. Expressed in terms of xylose the total furfural-producing material in B represents about 7 mg./4 hr. of which about two-thirds are destroyed by NaOI. The nature of the furfural-producing material has not been definitely determined. Although some of it may be glucuronic acid which French workers [see Cammidge, 1913; 1923] have long maintained is responsible for the non-fermentable reducing material of urine, a considerable part of it at least is probably pentose, since three yeasts, *M. tropicalis*, *M. pinoyi* and *M. metalondinensis*, which were found to have the ability to remove small amounts of pentose (xylose, arabinose, ribose and rhamnose), removed reducing material from urine fractions which had been freed from fermentable hexoses by *S. marxianus*, which does not attack pentose. Fraction G (the copper-lime filtrate) could not be analysed by mycological methods as it contained small amounts of material which were removed erratically by all the yeasts including baker's yeast.[1] However, yeast treatment of Fractions H and J shows "pentose"-like material which is equal in amount in the two fractions. That NaOI treat-

[1] This finding supports the conclusion of Harding & Selby [1933] that normal urine contains small amounts of reducing substances other than glucose which are removable by yeast.

ment did not destroy this material in Fraction G may be due to ketonic properties or to protection from oxidation by combination with some hydrolysable radical. Van Ekelen *et al.* [1933], Birch [1934] and many later workers have presented evidence showing the presence of ascorbic acid in urine, but the alkalinity of the clearing reagents and the low recovery of reducing material after copper-lime precipitation of ascorbic acid preclude the possibility that any appreciable fraction of the furfural-producing material in our fractions can be derived from vitamin C. That hydrolysis of Fraction G liberates reducing materials which on separation by copper-lime precipitation show properties similar to those in Fraction B suggests that the precursors are unfamiliar substances since the higher polysaccharides are removed by the preliminary clearing and mono- and di-saccharides are precipitated by copper-lime. The resistance of part of the furfural-producing material to NaOI is not due to protection from oxidation by a hydrolysable radical as Fraction F gives rise to fully as much furfural as does Fraction D. It is probable therefore that the furfural comes from ketonic material. Of the NaOI-resistant material a small amount, representing in most urines less than 1·0 mg./4 hr., can be accounted for by the presence of small amounts of fructose, as revealed by mycological analysis. A considerable portion of the remainder, over 2 mg./4 hr. as xylose, is furfural-producing material.

It would appear probable that the reducing material destroyed by the NaOI would be similarly affected by Br oxidation after 9–16 hr. Br treatment however causes an increase in the reducing power of, or the production of new reducing substances from, certain components of the urine fractions. This is quite apparent when the curve produced by Fraction B is compared with that from Fraction D, where all the free aldose has been destroyed by NaOI. In Fraction B there is a rapid drop in reducing power followed by a steady rise but in Fraction D no drop occurs, but only a rise which is very rapid in the first few hours and which reaches completion at the end of 8 days. Everett *et al.* [1934, 1] have ascribed the rise in the reducing value of whole urine produced by bromination to acid hydrolysis, but this could not be an important factor in the urine fractions studied here because no decrease in *p*H occurred and the rise in Cu reducing value took place in Fraction F where the sugars released by vigorous acid hydrolysis had been destroyed. It should also be noted that all the substances liberated by such acid hydrolysis acted as aldose towards NaOI and Br, so that if acid hydrolysis were to occur during bromination the products would be promptly destroyed. It has also been observed that with lactose and maltose, and also in the case of acid-hydrolysable urine sugar, both Br and NaOI oxidize the free reducing groups and subsequent acid hydrolysis liberates the protected reducing group. In the case of maltose and lactose such reducing sugar is liberated in theoretical amounts by acid hydrolysis after Br oxidation and is identified by mycological analysis as glucose and galactose respectively. Sucrose is not affected by either Br or NaOI.

It seems that during bromination one or probably more reducing substances resistant to oxidation by Br are formed or liberated, either by the action of Br on some substance which previously had no reducing value or by an increase in the reducing power of some substance originally only feebly reducing. The reducing value is increased to approximately $2\frac{1}{2}$ times the reduction due to substances left after NaOI, as can be seen by examination of the Br oxidation curve of Fraction D, in which all the free aldose has been destroyed by NaOI. Comparison of Fractions D and F shows that there are two reactions tending to increase the reducing substances, one which is relatively rapid and which is almost completed in 24 hr. and one which is much slower and which is not

complete until after 1 week. The second reaction does not take place in Fraction F. That is, hydrolysis permits the NaOI to destroy the substance which on Br treatment gives rise to a slow increase in reducing power. This, and the fact that acid hydrolysis (Fraction C) does not give rise to this substance would indicate that the increase in reducing power is more likely to be due to the production of a new compound than to the liberation of one already present.

Presumably these reactions are taking place in Fractions B, C and E during the first 6 hr. of Br treatment, but their effect is obscured by the much more rapid destruction of aldose. The shape of the rising curve after 12 hr. suggests that by the extension of this latter part back to zero time the curve would fall in the case of Fraction B near the starting-point of D, and in the case of C, near that of F. It is probable that a considerable part of the initial rise observed by Everett *et al.* in whole urines is due to the same compound which causes the rise in reduction in Fractions D and F and that this latter reaction is not caused by acid hydrolysis. That an initial rise does not occur in cleared urines is due at least partly to the fact that such solutions are much more readily saturated with Br than are whole urines, which contain large amounts of halogen-absorbing materials. Whereas the increase in copper-reducing ability of the Br-resistant fraction is almost as rapid in low concentration of Br as in a saturated solution, the oxidation rate of aldoses is affected greatly by Br concentration. Similar increases in reducing power were observed in filtrates from individual urines cleared with $HgSO_4$-KOH according to the directions of Everett *et al.* [1934, 2]. An extreme example of this is shown in Fig. 1. Other samples gave much smaller rises and a few gave curves similar to those described by Everett *et al.* [1934, 1].

As in the case of NaOI, Br oxidation leaves appreciable amounts of furfural-producing material.

It is evident that NaOI and Br oxidations are not of equal value in determining the ratio of aldose to non-aldose reducing substances in urine, but that owing to the fact that Br treatment gives rise to new reducing substances the latter type of analysis may give a false impression of the amount of non-oxidizable material. It would appear probable that the Br-resistant reducing material termed by Everett *et al.* [1934, 1] uroketose is not a single substance but is composed, in so far as those portions which are precipitated by copper-lime are concerned, of (*a*) Br- and NaOI-resistant reducing substances already present in urine and (*b*) new reducing material produced by the action of Br. It is probable that a part at least of (*a*) is a ketopentose, as evidenced by the production of furfural, considered with the action of the pentose-removing yeasts.

Summary

1. By the use of basic Pb acetate, $HgSO_4$ and a copper-lime precipitation method, a fraction of urine free from N and containing a high percentage of true sugars was obtained.

2. The changes in reducing power induced by NaOI and Br oxidations were observed.

3. Br oxidation gives rise to the production of new reducing substances resistant to further Br and NaOI oxidation and to yeast fermentation.

4. NaOI oxidation gives rise to little of any such substance and shows that 75% of the reducing material behaves as does aldose.

5. Evidence is presented which suggests the presence of small amounts of pentose in urine, some of which is ketonic in nature.

The writers wish to express their gratitude to the late Prof. V. J. Harding for helpful suggestions and criticism during the early stages of the work.

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LXIV. THE FATE OF CALCIUM AND MAGNESIUM AFTER INTRAVENOUS ADMINISTRATION TO NORMAL PERSONS

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Fifteen years ago there would seem to have been no doubt in the minds of physiologists that the divalent and trivalent metals were excreted mainly by the gastro-intestinal tract and that variations in the rate of this excretion maintained the normal levels within the body. This is still the opinion of most people who have not studied the subject, but within the last 10 or 12 years evidence has been collected which makes it essential to reconsider the whole question.

First, it has been shown that iron is certainly excreted much less freely than was previously supposed [McCance & Widdowson, 1937, 1]. It is, in fact, impossible to demonstrate any gastro-intestinal excretion of iron by man [Widdowson & McCance, 1937; McCance & Widdowson, 1938], although it is agreed that traces of this metal must get into the intestine from abraded cells and glandular secretions. It has, therefore, been suggested that the amount of iron in the body must be regulated by controlled absorption [McCance & Widdowson, 1937, 2] and this seems to have been supported by the most recent evidence [Hahn *et al.* 1938]. Secondly, much recent work on Ca has tended to show that in normal circumstances its excretion into the gastro-intestinal tract is negligible or at least plays no part in regulating the amount in the body [Christiansen, 1936; Aub *et al.* 1937; Henry & Kon, 1939]. The best evidence has been obtained (*a*) by the direct determination of the Ca excreted into the isolated large intestines of dogs [Nicolaysen, 1934] and men [Welch *et al.* 1936; Johnson, 1937], (*b*) by the determination of Ca balances under experimental conditions usually involving the parenteral administration of the metal [Nicolaysen, 1934; Christiansen, 1936; Albright & Sulkowitch, 1938]. Against this view, however, must be set certain rather older experiments such as those of Greenwald & Gross [1925] on dogs, Ross & Scriver [1928] and Pugsley [1932] on rats. From a study of faecal pellets Cowell [1937] also considers that rabbits excrete Ca into their large intestines. This excretion may be peculiar to the rabbit and in any case it probably has no regulatory function [Christiansen, 1936]. If the balance experiments which have been carried out on normal persons and on hyperthyroid patients are correct, relatively large quantities of Ca may be passed into the human gastro-intestinal tract, even when the food contains very little [Bauer *et al.* 1929; Tibbetts *et al.* 1932; Tibbetts & Aub, 1937, 2]. There is nothing to suggest however that this excretion has any regulatory function, but nevertheless, it requires further investigation. Thirdly, what little work has been carried out on Mg has also tended to show that this metal is not excreted in more than very small quantities into the gastro-intestinal tract [Ferguson & Ivy 1936; Nicolaysen, 1936].

In contrast to this it still appears true to say that certain non-physiological heavy metals are excreted predominantly by the gastro-intestinal tract. Thus,

lead and radium have been shown to be so excreted when they have been given parenterally or when they have been mobilized from the bones by parathormone [Hunter & Aub, 1926–7; Schlundt & Failla, 1931; Aub, 1935; Aub *et al.* 1938]. Mercury has been found to be excreted—slightly at any rate—in the faeces after parenteral administration [Young *et al.* 1930]. The site of the excretion of these metals, however, is not yet known. Histological attempts have been made to solve the problem [Schönlebe, 1937], but physiological experiments should be undertaken, similar to those which have already been carried out for Ca and Fe on animals with isolated large intestines.

The experiments now to be described were undertaken in conjunction with work on the excretion of Fe [Widdowson & McCance, 1937; McCance & Widdowson, 1938]. They are, it is believed, the first of their kind to be made on strictly normal persons, and the results are in keeping with the view that the amount of Ca or of Mg in the body is not regulated by glands which secrete into the gastro-intestinal tract.

Experimental arrangement and technique

The work was carried out at King's College Hospital, London, on the same six persons already described [McCance & Widdowson, 1938]. Balance experiments were performed at two different (physiological) levels of intake by mouth and finally during a time of parenteral administration. Each balance experiment lasted 14 days and was preceded by a preliminary period of 2 or 3 days. The dietary regime was continued beyond the 14 days until the last specimen of faeces had been collected. Distilled water was used for drinking purposes and in the preparation of beverages. The use of toothpaste was avoided. The increased oral intake of Ca was brought about by increasing the intake of milk and that of Mg by administering medicinal supplements. Both metals were injected intravenously as the gluconates; the method of so doing was described by McCance & Widdowson [1938]. 0·186 g. of Ca and 0·219 g. of Mg were given daily. Even run in slowly over a period of 30–45 min. the intravenous injection of these amounts to normal persons is rather an uncomfortable experience, for it is accompanied by constant flushings and sometimes by nausea, but no further difficulties were encountered except that one subject (R. M. L.) fainted at the close of his 7th injection. The management of the food, drinks, urine and faeces has been described by McCance & Widdowson [1938] and the chemical methods employed were those described by McCance *et al.* [1936].

Results

(1) *Calcium*

Table I shows the results for the six subjects at the lower levels of intake which varied from 6·43 to 9·20 g. Ca in the 14 days—an average of 0·582 g. Ca/subject/day. R. A. M. and R. H. E.'s balances were rather unaccountably negative but the summarized results amounted to a negative balance of only 2·03 g. on an intake of 49 g.

Table II gives the results at the higher levels of intake by mouth which now varied from 8·10 to 12·88 g. Ca in the 14 days and averaged 0·734 g. Ca/subject/day. R. A. M. and R. H. E. were still in negative balance and it should be pointed out that these negative balances cannot be explained by incomplete collections of faeces for at this stage both subjects were in positive iron balance [McCance & Widdowson, 1938]. The summarized results showed the group to be practically in balance.

Table I. *Ca balances at the lower levels of intake*

Subject	Ca intake from food, g.	Ca output, g. Urine	Faeces	Total	Ca balance, g.
E. M. W.	9·20	1·34	7·50	8·84	+0·36
M. M.	8·61	2·20	6·86	9·06	−0·45
H. L.	6·43	0·64	5·72	6·36	+0·07
R. A. M.	7·82	2·49	6·37	8·86	−1·04
R. M. L.	8·84	2·81	5·75	8·56	+0·28
R. H. E.	7·98	2·50	6·73	9·23	−1·25
Totals	48·88	11·98	38·93	50·91	−2·03

Table II. *Ca balances at the higher levels of intake*

Subject	Ca intake from food, g	Ca output, g. Urine	Faeces	Total	Ca balance, g.
E. M. W.	11·35	1·70	9·81	11·51	−0·16
M. M.	11·92	2·47	9·40	11·87	+0·05
H. L.	8·10	0·87	7·10	7·97	+0·13
R. A. M.	8·97	2·42	7·10	9·52	−0·55
R. M. L.	12·88	3·53	9·12	12·65	+0·23
R. H. E.	8·46	2·70	6·46	9·16	−0·70
Totals	61·68	13·69	48·99	62·68	−1·00

Table III contains the results when Ca was administered intravenously. The oral intakes varied from 7·09 to 9·78 g. Ca in the 14 days—an average of 0·628 g. Ca/subject/day. Neglecting for the moment individual results, it is evident that additional Ca, equivalent to a very great part if not the whole of the Ca administered intravenously, had been excreted before the experiment came to an end, since the summarized results for the group indicated a positive balance of only 2·52 g. on an intake of 68·31 g. Unfortunately it is not possible to define the limits of error in an experiment of this kind, but from the individual results it would appear that R. M. L. and possibly R. H. E. had not excreted as much additional Ca as had been injected into them by the end of the experiment—but that the others had certainly done so.

Table III. *Ca balances during intravenous administration of the metal*

Subject	Ca intake, g. From food	From injection	Total	Ca output, g. Urine	Faeces	Total	Ca balance g.
E. M. W.	8·54	2·60	11·14	3·37	7·37	10·74	+0·40
M. M.	9·00	2·60	11·60	5·04	6·59	11·63	−0·03
H. L.	7·09	2·60	9·69	2·74	6·27	9·01	+0·68
R. A. M.	9·78	2·60	12·38	5·21	8·11	13·32	−0·94
R. M. L.	9·40	2·60	12·00	4·75	5·92	10·67	+1·33
R. H. E.	8·90	2·60	11·50	4·17	6·25	10·42	+1·08
Totals	52·71	15·60	68·31	25·28	40·51	65·79	+2·52

Table IV shows the ratios of urinary/faecal Ca and faecal/food Ca of all the subjects during each stage of the experiment. The relative constancy of the urinary/faecal ratio at both levels of intake by mouth suggests that during the period of high Ca intake the metal was divided between the urine and faeces in about the same proportions as it had been at the lower level of intake. The large increase in the ratio during intravenous administration indicates that in these circumstances most of the additional Ca was being excreted in the urine. In

Table IV

Subject	Ratios Urinary Ca/Faecal Ca: Lower intakes	Higher intakes	Injection stage	Ratios Faecal Ca/Food Ca: Lower intakes	Higher intakes	Injection stage
E. M. W.	0·18	0·17	0·46	0·82	0·87	0·86
M. M.	0·32	0·26	0·77	0·80	0·79	0·73
H. L.	0·11	0·12	0·44	0·89	0·88	0·88
R. A. M.	0·39	0·34	0·64	0·81	0·79	0·83
R. M. L.	0·49	0·39	0·80	0·65	0·71	0·63
R. H. E.	0·37	0·42	0·67	0·84	0·76	0·70
Average	0·31	0·28	0·63	0·80	0·80	0·77

fact, since there was no rise in the faecal/food Ca ratios during this time, it is clear that all the additional Ca, equivalent to the whole or almost the whole of the amount injected, must have been excreted by the kidney.

It is possibly of some interest to note that although the urinary/faecal Ca ratio is remarkably constant in any one individual it varies considerably from one individual to another. The findings of Owen [1939] in a study of 10 adult and elderly males were very much the same. Thus about 30 % of R. M. L.'s total output of Ca was found in the urine, whereas only about 10 % of H. L.'s. It would be an interesting problem to find out why this should be so. In the light of the experiments in which Ca was given intravenously, it would look as though H. L. consistently absorbed less Ca than did R. M. L. It may further be noted that the three female subjects (E. M. W., H. L., and M. M.) had the three lowest urinary/faecal Ca ratios, but it is impossible to say whether this represents a real sex difference since the number of subjects was so small. The work of Owen [1939] suggests that occasionally males may excrete as little as 10–11 % of their total Ca in the urine.

(2) *Magnesium*

Table V shows the results for the six subjects at the lower levels of intake, which varied from 3·21 to 4·44 g. Mg in the 14 days, an average of 0·271 g. Mg/person/day. On those amounts of Mg all the subjects were in fair balance and the summarized results amounted to an insignificant positive balance of 0·22 g. on an intake of 22·8 g.

Table V. *Mg balances at the lower levels of intake*

Subject	Mg intake from food, g.	Mg output, g.: Urine	Faeces	Total	Mg balance, g.
E. M. W.	3·40	1·51	2·25	3·76	−0·36
M. M.	3·87	1·44	2·36	3·80	+0·07
H. L.	3·21	1·07	2·10	3·17	+0·04
R. A. M.	4·44	1·74	2·52	4·26	+0·18
R. M. L.	3·98	1·81	1·95	3·76	+0·22
R. H. E.	3·90	1·71	2·12	3·83	+0·07
Totals	22·80	9·28	13·30	22·58	+0·22

Table VI indicates the results on the higher levels of intake by mouth, which varied from 5·18 to 6·38 g. Mg in the 14 days and averaged 0·405 g. Mg/subject/day. All the subjects were again in very fair balance and the summarized results again amounted to a small positive balance of 0·73 g. on a total intake of 34 g.

Table VI. *Mg balances at higher levels of intake*

Subject	Mg intake, g. From food	From medicine	Total	Mg output, g. Urine	Faeces	Total	Mg balance g.
E. M. W.	3·51	1·76	5·27	1·71	3·41	5·12	+0·15
M. M.	3·57	1·76	5·33	1·18	3·88	5·06	+0·27
H. L.	2·78	2·40	5·18	1·38	3·84	5·22	−0·04
R. A. M.	3·98	2·40	6·38	1·85	4·23	6·08	+0·30
R. H. L.	3·79	1·76	5·55	2·18	3·45	5·63	−0·08
R. H. E.	3·90	2·40	6·30	2·18	3·99	6·17	+0·13
Totals	21·53	12·48	34·01	10·48	22·80	33·28	+0·73

Table VII gives the results when Mg was administered intravenously. The oral intakes ranged from 3·20 to 4·28 g. in the 14 days, and averaged 0·279 g. Mg/subject/day. 3·06 g. Mg were given intravenously to each subject (0·219 g./day). It is evident from the balances that, as with Ca, additional Mg equal to practically the whole of the quantity given intravenously had been excreted before the experiment terminated. The summarized results showed a positive balance of 2·19 g. on a total intake of 41·78 g. The individual results suggest that M. M. was the only subject who had not got rid of the injected Mg.

Table VII. *Mg balances during the intravenous administration of the metal*

Subject	Mg intake, g. From food	From injection	Total	Mg output, g. Urine	Faeces	Total	Mg balance g.
E. M. W.	3·75	3·06	6·81	4·53	2·10	6·63	+0·18
M. M.	3·79	3·06	6·85	3·39	2·15	5·54	+1·31
H. L.	3·20	3·06	6·26	3·77	2·21	5·98	+0·28
R. A. M.	4·14	3·06	7·20	3·98	2·47	6·45	+0·75
R. M. L.	4·26	3·06	7·32	4·75	3·16	7·91	−0·59
R. H. E.	4·28	3·06	7·34	4·77	2·31	7·08	+0·26
Totals	23·42	18·36	41·78	25·19	14·40	39·59	+2·19

Table VIII

Subject	Ratios Urinary Mg/Faecal Mg Lower intakes	Higher intakes	Injection stage	Ratios Faecal Mg/Ingested Mg Lower intakes	Higher intakes	Injection stage
E. M. W.	0·67	0·50	2·16	0·66	0·65	0·56
M. M.	0·61	0·30	1·58	0·61	0·73	0·57
H. L.	0·51	0·36	1·70	0·65	0·74	0·69
R. A. M.	0·69	0·44	1·62	0·57	0·66	0·60
R. M. L.	0·93	0·63	1·50	0·49	0·62	0·74
R. H. E.	0·81	0·55	2·07	0·54	0·63	0·54
Average	0·70	0·46	1·77	0·58	0·67	0·62

Table VIII gives the ratios of urinary/faecal Mg and faecal/food Mg of all the subjects during each stage of the experiment. The fall in the urinary/faecal ratio during the higher oral intakes indicates that excretion of the additional Mg was not shared in the previous proportions between the urine and faeces. Tibbetts & Aub [1937, 1] also found this to happen but their Mg supplements were much larger. Indeed, a comparison of the summarized results of Tables V and VI suggests that the kidney excreted very little of the extra Mg taken by mouth, and in this respect the results differ from those obtained with Ca. In all probability very little of the extra Mg taken by mouth was absorbed. The large increase

in the urinary/faecal ratio during the fortnight of intravenous administration indicates that, as in the case of Ca, the kidney was excreting much of the extra Mg, and a study of the food/faecal ratios shows that, as with Ca, none of the additional Mg was finding its way into the faeces.

Discussion

These experiments are only a small contribution towards the solution of a very large and complicated problem, but they raise interesting points which deserve consideration. They would seem to show, first, that the injection of Ca or Mg intravenously into normal persons provokes no increased excretion of these metals by the gastro-intestinal tract, and taken in conjunction with previous work they support the view put forward earlier in this paper that the gastro-intestinal tract does not excrete these metals in amounts which vary with the levels in the plasma or with the needs of the body. It would be very interesting to be able to say why lead and radium are treated so differently. Secondly, these experiments show quite clearly that Ca and Mg given intravenously to normal persons are rapidly excreted by the kidney, and it follows that the normal kidney must be exceedingly sensitive to variations in the levels of these metals in the serum. This mechanism must always be in operation and should be seriously considered whenever variations in the urinary excretion of these metals have to be explained. It would seem from the literature, however, that in hyperthyroidism and sometimes in hyperparathyroidism there may be a large increase in the output of Ca in the urine without a corresponding increase in the serum Ca, so that other factors are probably involved, at any rate in the case of this metal. In the absence of an acidosis in these diseases, which might be expected to raise the urinary excretion of these metals by increasing the concentration of ultrafilterable ions in the serum, this must be due to a change in the activity of the kidney itself. It would be most interesting and valuable to know in what way secretions of the thyroid and parathyroid affected the kidney.

Summary

1. 0·186 g. Ca and 0·219 g. Mg were injected intravenously into six normal persons every day for a fortnight.

2. This led to a rapid increase in the excretion of Ca and Mg by the kidney and in most subjects the additional output was equal to the amount injected.

3. No evidence was found that any additional Ca or Mg was excreted into the gastro-intestinal tract.

The authors very much appreciated the co-operation of their fellow subjects, and of Mrs Ellis who was responsible for much of the dietary side of the experiment. The Medical Research Council paid a large part of the expenses and E. M. W. is indebted to the Council for a personal grant. The gluconates were obtained from "Sandoz" Products, Ltd., and the magnesium salt was very kindly presented by the Company free of charge.

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LXV. ANALYSIS OF PROTEINS

XI. THE PRODUCTS OF THE ACTION OF SODIUM HYDROXIDE ON CASEINOGEN. THE COMPOSITION OF DEPHOSPHOCASEIN, OR DEPOCASEIN

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Plimmer & Bayliss [1906] first showed that the P of caseinogen was completely separated as phosphoric acid by the action of 1 % NaOH at 37° in 24 hr. They also noticed that the solution became very milky in appearance and that acidification precipitated a substance resembling caseinogen. Rimington & Kay [1926] confirmed these and other observations of Plimmer & Bayliss and called the substance thrown down by acetic acid "dephosphorized caseinogen", although it represented only about 40 % of the caseinogen. Rimington [1927] analysed dephosphorized caseinogen by the Van Slyke method and noticed little difference in composition from that of caseinogen except in amide-N. No other work upon this substance has since been published. Meanwhile new and improved methods of analysis of proteins have been devised, and they have now been used in further examination of this substance. Though our results are incomplete, this substance has resemblances with caseinogen, but must be considered different. The term "dephosphorized caseinogen" chosen by Rimington & Kay is not altogether appropriate since caseinogen is converted by 1 % NaOH into this product and a substance or mixture of substances resembling a primary proteose in about equal parts. The latter is a caseose and as it does not contain P is a dephosphocaseose, or shortly depocaseose. The former resembles a metaprotein in solubilities in 0·1 *N* alkali and acid and caseinogen in being precipitated by more acid. It might be called metacasein. This term does not indicate the absence of phosphorus. It is a dephosphometacasein, or for brevity dephosphocasein or depocasein.

It was hoped that chemical examination of the intermediate products of hydrolysis of proteins would show a less complex mixture of amino-acids, so that ultimately it might be possible to fill in the gaps in the amino-acid composition and find out in which order the amino-acids were combined. In the case of caseinogen it appears possible that the whole molecule is made up of two or more very similar portions. Sörensen [1930] adopted the view that caseinogen consisted of many similar molecules of the same solubility.

Experimental

Light white soluble casein. L.W.S. casein

Light white soluble casein supplied by The British Drug Houses, Ltd., has been used for the preparation of depocasein. Dr F. H. Carr kindly informed us that this material is the sodium salt of caseinogen and is prepared by allowing fat-free milk to develop lactic acidity and then precipitating caseinogen by the

addition of very dilute HCl. The caseinogen is washed with water and converted into the sodium salt. Two separate batches supplied at different times gave the following percentage analyses:

Batch	H_2O lost at 105°	N	P	S	Ash	Na	Corrected for H_2O and Na contents: N	P	S
1	9·9	12·6	0·72	0·42	3·9	1·6	14·2	0·81	0·47
2	10·1	12·5	0·67	0·59	3·5	1·4	14·1	0·76	0·66

The computation of the amount of Na in the ash is based upon the figures for P and S as follows:

0·72 % P corresponds with	3·7 % Na_3PO_4	or 3·0 % $Na_4P_2O_7$	or 2·3 % $NaPO_3$
containing Na =	1·56 %	or 1·04 %	or 0·52 %
and leaving Na_2CO_3 =	0·2	or 0·9	or 1·56
containing Na =	0·09	or 0·39	or 0·7
Total Na in ash	= 1·65	or 1·43	or 1·22
0·42 % S corresponds with	1·78 % Na_2SO_4	containing	0·58 % Na
Remainder of ash as Na_2CO_3	2·12 %	containing	0·92 % Na
Total Na	—	—	1·5 %

If the ash were pure Na_2CO_3 = 3·9 %, it would contain 1·70 % Na. The Na content of the L.W.S. casein is thus about 1·6 %; similarly for batch 2 with 3·5 % ash, the Na is about 1·4 %.

Preparation of depocasein

The conditions for precipitating depocasein from the alkaline digest of caseinogen were not mentioned by Plimmer & Bayliss. Rimington & Kay simply stated that acetic acid was added to give maximum precipitation. In order to determine the optimum amount of acetic acid for precipitation 5 g. of L.W.S. casein were dissolved in water, 62·5 ml. of *N* NaOH added, the solution brought to 250 ml. and placed in an incubator at 37°. After 24 hr. the solution was cooled till it occupied the original volume. 10 ml. were removed to determine the total N, and five lots of 25 ml. were placed in Kjeldahl digestion flasks. Varying volumes of 25 % v./v. (4·32 *N*) acetic acid were added to precipitate the depocasein. The sticky precipitates adhered to the bottom and sides of the flasks. After 24 hr. the contents of each flask were filtered and the precipitates washed with 20 ml. of 0·5 % acetic acid. The filters were returned to their respective flasks and N determinations carried out with the following results:

Total N of the digested solution = 2·49 g./l.

ml. acetic acid added to 25 ml.	4·20	4·30	4·40	4·50	4·60	4·70
N pptd. in g./l.	1·28	1·32	1·36	1·30	1·29	1·28

The maximum yield of depocasein was thus obtained with 4·40 ml. and the precipitate contained $\frac{1{\cdot}36 \times 100}{2{\cdot}49} = 55$ % of the total N.

Depocasein was prepared in large quantities by dissolving 40 g. L.W.S. casein by grinding with water, placing in a Winchester bottle, adding 500 ml. *N* NaOH, diluting to 2 l., warming to 37° and maintaining at this temperature for 24 hr. The solution was then transferred to a large beaker and 87 ml. of glacial acetic acid run in from a burette during 5–10 min. with constant stirring. Most of the precipitate adhered to the beaker; the solution was poured off after 24 hr. and subsequent precipitations carried out on top of the first. When sufficient had been collected, usually from four bottles, the precipitates were redissolved by grinding with cold *N* NaOH and immediately reprecipitated with dilute H_2SO_4, the acid being added until the precipitate aggregated and the mother liquor gave no further precipitate with acid. The depocasein was filtered, washed with water and dried with successive changes of absolute alcohol and

finally in a vacuum desiccator over H_2SO_4. The product was a fine white powder and the yield approximately 40 % of the L.W.S. casein taken, some having been lost during purification. Two separate batches of about 250 and 500 g. gave the following percentage analytical figures:

						Corrected for H_2O and Na		
Batch	H_2O	N	P	S	Ash Na	N	P	S
1	19·7	10·90	0·025	0·34	0·7=0·3	13·6	0·03	0·43
2	10·1	11·97	0·030	1·08	0·7=0·3	13·4	0·03	1·20

The S content of batch 2 is erroneous. Analyses of further batches of depocasein by Mr T. J. R. Macara showed 0·42 and 0·45 % S. The low P content compares with the similar figure of Rimington & Kay. The N content is lower than that of caseinogen owing to removal of amide-N by the action of NaOH.

Depocasein dissolves easily in 0·1 *N* NaOH and 0·1 *N* HCl and is precipitated on neutralization and by addition of 2 *N* HCl. The solution in 0·1 *N* alkali made just neutral to incipient precipitation with 0·1 *N* H_2SO_4 gives gelatinous precipitates with silver nitrate, lead acetate, copper sulphate and ferric chloride solutions. With mercuric chloride there was no precipitate till 2 *N* HCl was added. Mercurous nitrate gave a white precipitate which rapidly became black. Calcium chloride, barium chloride and magnesium sulphate solutions did not give precipitates.

The filtrate from depocasein is almost completely precipitated by half saturation with ammonium sulphate, but appears to contain a mixture of proteoses. On evaporating a volume of filtrate containing 15·8 g. N *in vacuo* at 45°, by which some of the acetic acid was removed, a syrup containing sodium acetate remained. The crystals dissolved on diluting with water leaving an insoluble gelatinous mass. The liquid was poured off and the mass washed with water and allowed to dry. It was found to be insoluble in water and alcohol, but soluble in a range of mixtures of water and alcohol. It weighed 28 g. and contained 10·8 % N. The liquid after concentration was fractionated with alcohol. The first fraction weighing 2 g. was insoluble in water. A second fraction of 14·5 g. was thrown out at about 60 % concentration of alcohol and a third fraction of 8·9 g. at over 90 % alcohol. Both the latter were soluble in water and the third fraction was hygroscopic.

Distribution of N

L.W.S. casein, depocasein, its filtrate and the three fractions, also a depocasein made after 144 hr. treatment with 1 % NaOH were examined for distribution of N by Van Slyke's method by the procedure described by Plimmer & Rosedale [1925] using 3 g. (2 g. of fraction 1) of protein in each analysis. The results, the mean of two determinations, are shown in the following table together with the data of Rimington & Kay (R. & K.) for dephosphorized caseinogen:

	L.W.S. casein		Depocasein			Fractions			144-hr.
	Batch 1	Batch 2		(R. & K.)	Filtrate	1	2	3	depocasein
Amide-N	11·1	11·6	5·3	4·4	1·9	4·4	7·1	5·5	0·2
Humin-N	1·2	2·0	0·9	1·8	3·2	1·6	1·2	1·7	0·1
Arginine-N	8·0	11·6	8·8	6·8	7·4	7·5	6·9	9·0	9·0
Histidine-N	5·1	2·3	8·8	7·0	8·2	10·4	0	4·9	6·3
Lysine-N	7·9	11·9	9·8	13·4	8·8	9·1	13·8	11·0	14·7
Monoamino:									
Amino-N	60·0	56·8	65·0	60·2	49·0	60·7	65·4	67·9	65·8
Non-amino-N	2·5	4·0	0	3·9	14·3	5·7	5·0	0	0·7
Total N recovery	101·8	100·2	98·6	97·5	92·8	99·4	99·4	100·0	96·8
Total N of solid	14·2	14·1	13·6	—	—	10·8	11·4	6·8	—

In the first place our figures do not entirely agree with those of Rimington & Kay. The most noticeable differences are those for lysine-N and the non-amino-N of the monoamino fraction. Our figure is erroneous as proline has actually been isolated (below). The absence or low content of non-amino-N in depocasein is accounted for by its high value in the filtrate, but the fractions isolated did not have such high amounts.

The amide-N of the filtrate is after determination of ammonium salt by distillation *in vacuo* with baryta or sodium carbonate and alcohol and recalculation to a total N of $100-19=81$ % of the original total N of the filtrate.

Of the bases, arginine-N is about the same in L.W.S. casein, depocasein and the filtrate. Histidine appears to be greater in depocasein than in L.W.S. casein. The lysine-N, which is dependent on the accuracy of the determination of the other bases, is variable. There is unaccountable variation in the figures of the monoamino fractions.

Determination of arginine, histidine and lysine

Three separate analyses of both L.W.S. casein and depocasein for the amounts of the three basic amino-acids were made by the improved methods of Vickery and his co-workers as described in several separate papers [1926–33]. A final description of the best procedure was not published. The procedure adopted in these analyses was briefly the following.

Hydrolysis. 50 g. of protein were hydrolysed with 240 ml. conc. HCl and 160 ml. water for 30 hr. HCl was partly removed by three evaporations *in vacuo* and then completely by precipitation with Ag_2O and H_2SO_4 at pH $< 2{\cdot}8$. The AgCl was extracted with dilute HCl and then with water, and chlorides again removed from the extract. The chloride-free solution and extracts were evaporated, brought to 2 l. and total N estimated in 10 ml. to ascertain the amount of protein present.

Precipitation of histidine and arginine. After concentrating to 1 l. excess of conc. $AgNO_3$ solution was added, as shown by testing a portion with baryta solution, and hot baryta solution added to pH 12 (alizarin blue S). The silver precipitate of arginine and histidine was filtered off next day, and without washing suspended in water, adjusted to pH 2·8 with H_2SO_4 and decomposed with H_2S. The filtrate and washings from Ag_2S were evaporated *in vacuo*, brought to 500 ml. and N determined on 10 ml.

Histidine. Histidine silver was precipitated by adding Ag_2O and H_2SO_4 alternately until excess of silver was present keeping the reaction below pH 2·8 and then cautiously adding cold baryta solution until the reaction was between pH 7·2 and 7·8, as shown by the greenish colour of a drop of bromophenol blue on the surface and flocculation of histidine silver. The precipitate was again dissolved and thrown out at a reaction as near as possible to pH 7·4. The histidine silver was filtered off next day and washed once with water. It was suspended in water, made acid with H_2SO_4 and decomposed with H_2S. The solution was evaporated and brought to 250 ml. 5 ml. were used for N estimation and the remainder treated with an equal volume of 10 % $HgSO_4$ in $2N$ H_2SO_4. The precipitate of histidine mercuric sulphate after washing with 2·5 % $HgSO_4$ in N H_2SO_4 was suspended in water and decomposed with H_2S. The solution and washings were evaporated and brought to 500 ml. N was determined in 5 ml. and the remainder concentrated and treated with a slight excess of flavianic acid (14·96 g. for 1 g. histidine N). Histidine diflavianate crystallized out and a

further crop was obtained from the filtrate. Both crops were recrystallized, the pure crystals dried at 100° and weighed. They had a decomposition point of 235°.

Arginine. The filtrates from the two precipitations of histidine silver were combined, acidified with H_2SO_4, evaporated *in vacuo*, brought to 1 l. and N determined in 20 ml. The remainder, tested for excess of Ag which was present, was treated with hot baryta solution to *p*H 12. The precipitate of arginine silver was filtered off, washed twice with baryta solution, suspended in water, acidified with H_2SO_4 and decomposed with H_2S. The solution and washings from Ag_2S were concentrated, brought to 250 ml. and arginine-N estimated in 5 ml. To the remainder, heated to boiling, the requisite amount of flavianic acid (5·61 g. for 1 g. arginine-N) in hot water was added. Arginine monoflavianate crystallized out and a further crop of diflavianate was obtained from the filtrate. The two filtrates at *p*H 12 were combined, acidified, evaporated to a small volume and precipitation at *p*H 12 repeated. No further precipitation occurred showing that no arginine had escaped the first precipitation.

Lysine. The filtrates from the two precipitations at *p*H 12 containing lysine and other amino-acids, also baryta and silver, were acidified with H_2SO_4, saturated with H_2S and the precipitate removed and washed. The solution was neutralized with NaOH to Congo red and evaporated *in vacuo*. Ammonia was removed by making alkaline to phenolphthalein, adding 2 vol. alcohol and distilling *in vacuo*. After acidifying to *p*H 2·8, making to 500 ml. and estimating N in 10 ml., the remainder was acidified with H_2SO_4 to 5 % by weight and precipitated with 20 % phosphotungstic acid. The lysine phosphotungstate after 4 days in the dark was filtered off and washed with 2·5 % phosphotungstic acid in 5 % H_2SO_4, dissolved in a mixture of acetone and water and decomposed with hot baryta solution until the reaction was permanently alkaline to phenolphthalein. The filtrate and washings from Ba phosphotungstate were acidified with H_2SO_4, filtered from $BaSO_4$, concentrated and reprecipitated with phosphotungstic acid as before at a volume of 650 ml. After 24 hr. the lysine phosphotungstate was filtered off and recrystallized from 5 % H_2SO_4 containing 2·5 % phosphotungstic acid. After 4 days the crystalline lysine phosphotungstate was filtered off and decomposed as above with acetone and baryta. Acetone was removed by evaporation and Ba quantitatively with H_2SO_4. The solution and washings were brought to 250 ml. and N determined in 5 ml. The remainder was concentrated *in vacuo* to a thin syrup, alcohol added till slight precipitation occurred and saturated alcoholic picric acid solution carefully added till lysine picrate was completely thrown down. The precipitate was washed with alcohol and ether, dried in air and weighed.

With allowances made for aliquots removed for N determinations and for solubilities of histidine diflavianate, arginine monoflavianate and lysine picrate the quantities obtained were:

	L.W.S. casein			Depocasein		
	(1)	(2)	(3)	(1)	(2)	(3)
Arginine flavianate	3·20	3·75	3·91	3·20	3·20	3·73
Histidine diflavianate	0·97	2·71	3·46	2·07	3·23	2·66
Lysine picrate	3·84	Lost	4·52	4·49	3·03	3·44

The results of the analyses were thus variable and disappointing. In one case all the bulky inorganic precipitates were examined for N. Small quantities were retained, but extraction with HCl and repetition of the silver precipitations gave no further quantities of the diamino-acids.

The weights of basic amino-acid in the proteins calculated from the highest analytical figure gave the following percentage amounts:

	Depocasein	L.W.S. casein	Pure caseinogen [Vickery & White, 1933]
Arginine	3·32	3·15	3·85
Histidine	1·58	1·53	1·83
Lysine	4·49	4·52	6·25

In comparison with the data of Vickery & White [1933] all the data are lower. Those for lysine are undoubtedly too low. The discrepancy for lysine is attributable to the presence of nitrate in the lysine fractions which had usually to be left over for many days before making lysine phosphotungstate. During this time reduction to nitrite probably occurred with consequent destruction of part of the lysine. In the earlier paper by Vickery & Leavenworth the use of silver nitrate was condemned, but in the later paper by Vickery & Block its use was advised.

The molecular ratios of the amino-acids in the proteins obtained by dividing the percentage figures by the mol. wt. are:

	Histidine	Arginine	Lysine
L.W.S. casein	0·0100	0·0181	0·0272
Depocasein	0·0102	0·0191	0·0300

which work out, more particularly for depocasein, as histidine : arginine : lysine $= 1 : 2 : 3$.

Depocasein and L.W.S. casein from these analyses thus show no important difference in their contents of basic amino-acids. In comparison with Vickery and White's figure for lysine in pure caseinogen depocasein contains a smaller quantity.

Comparison of these data with those by Van Slyke's method by converting the N figures into weights of protein and amino-acids gives:

	L.W.S. casein		Depocasein	
	Vickery method	Van Slyke method	Vickery method	Van Slyke method
Arginine	3·15	5·08	3·32	3·72
Histidine	1·53	1·25	1·58	4·61
Lysine	3·98	8·75	4·38	6·95

There is little correspondence. Plimmer & Lowndes [1938], who made an examination of the basic amino-acids in the Van Slyke solution, were unable to give an explanation of this discrepancy. In the Van Slyke method there is no attempt at purification of the components in either the mono- or di-amino fractions. Arginine phosphotungstate is definitely not completely precipitated and yet the Van Slyke figure is higher. Monoamino-acids may be thrown down by phosphotungstic acid, particularly proline. The combination of these factors accounts for variability in the Van Slyke figures.

Determination of tyrosine and tryptophan

The method of Folin & Looney [1922] and its modification by Folin & Ciocalteu [1927] have been used for the determination of tyrosine and tryptophan in both L.W.S. casein and depocasein.

(*a*) *Folin & Looney method.* The results by this method for L.W.S. casein were very variable. Tyrosine values ranged from 1·35 to 3·76 %, and tryptophan values from 1·20 to 1·51 %.

From the consideration that the protein contains three times as much tyrosine as tryptophan, estimations of each amino-acid were made separately. For tyrosine, 2 or 3 ml. of the hydrolysate were precipitated with 2 ml. reagent. Colours comparable with standards of 1 mg. of tyrosine were secured and the results were concordant. For tryptophan, 6 or 8 ml. of hydrolysate were necessary. Complete precipitation of tryptophan mercuric sulphate was not effected with 2 ml. of reagent. Satisfactory results were obtained with 3 ml. reagent, and were not improved with 4 ml. Mercuric sulphate appears to combine with other amino-acids. 2 ml. reagent contain 200 mg. $HgSO_4$, a large excess over the amount required to throw down the tryptophan present. This inference is borne out on addition of the Na_2CO_3 solution; with the tyrosine standard there is precipitation of $HgCO_3$, with the hydrolysed solution the precipitate was small and sometimes absent. The mercuric amino compounds appear not to be easily decomposed by Na_2CO_3.

The best results by this modification for L.W.S. casein were:

Tyrosine 7·09 and 7·01 %
Tryptophan 1·77 and 1·76 %

(*b*) *Folin & Ciocalteu method.* The chief modifications of the Folin-Looney method consist in hydrolysis by means of 20 % NaOH, the use of increased quantities of $HgSO_4$ and phenol reagents, and the estimation of tyrosine by Millon's reagent. By this method the values for L.W.S. casein were tyrosine 6·7 % and tryptophan 0·36 %. The tyrosine value compares favourably with that by baryta hydrolysis, but the tryptophan value is about 1/5. Hydrolysis by 20 % NaOH seems to produce extensive decomposition of tryptophan. The stability of tryptophan to NaOH hydrolysis was therefore tested. Complete destruction occurred on boiling tryptophan alone, but on boiling with gelatin the destruction amounted to only 35 %.

(*c*) *Estimation by tryptic hydrolysis.* A solution of L.W.S. casein was submitted to hydrolysis by trypsin in faintly alkaline solution and tryptophan estimated at various times of digestion by the Folin-Ciocalteu procedure. The content of tryptophan was found to be 1·75–1·87 % indicating that baryta hydrolysis gives correct results.

(*d*) *Combination of the Folin-Looney-Ciocalteu methods.* Determination of tyrosine and tryptophan has been effected by baryta hydrolysis of 1 g. protein, and precipitation of the solution with 4 ml. of $HgSO_4$ reagent. Tyrosine was estimated both by the Millon reaction and the phenol reagent. Tryptophan was estimated by the phenol reagent and by bromination as described by Plimmer & Lowndes [1938]. The results calculated as percentage of dry mineral-free protein were:

	Tyrosine		Tryptophan	
	Millon reagent	Phenol reagent	Phenol reagent	Bromination
L.W.S. casein batch 1	—	7·09	1·77	—
	—	7·01	1·76	—
batch 2	5·26	5·19	1·21	1·36
Depocasein from batch 2	8·20	7·08	1·24	1·39
	8·05	7·31	1·23	1·39
	8·21	—	—	—
	8·15	7·20	—	—
Mean	7·68		1·31	

The two batches of L.W.S. casein gave different values for tyrosine and tryptophan, indicating a difference between them. This may be explained by its

method of preparation which involves production of lactic acidity by bacterial action. Tyrosine and tryptophan may be split off to a different extent for each batch. Further the commercial preparations may contain varying amounts of fat.

Folin & Ciocalteu give 6·41 % tyrosine and 1·4 % tryptophan in pure caseinogen.

Our figures for tyrosine in depocasein are higher by the Millon method than the phenol method. Preference is given to the phenol method, but the mean figure must be taken. Depocasein differs from caseinogen in containing more tyrosine. Tryptophan is the same in both proteins.

Determination of cystine and methionine

These amino-acids were estimated for us by Mr J. Lowndes by the latest method of Baernstein [1936] using the alteration in distillation mentioned by Plimmer & Lowndes [1938]. The results compared with caseinogen were:

	Depocasein		Caseinogen
Cystine	0·288		0·36 %
	0·313	0·30 %	
Methionine	2·077		
	2·110	2·09 %	2·89 %

The cystine contents of caseinogen and depocasein are thus the same, but the methionine content of depocasein is 0·8 % lower.

Determination of glycine

The colorimetric method of Patten [1935] for determination of glycine with *o*-phthalaldehyde was tried with both L.W.S. casein and depocasein. It gave the following results calculated on dry mineral-free protein: L.W.S. casein, 1·0 %; depocasein, 0·2 %. The figure for casein is twice that of Patten. The colour with depocasein was so slight that reproducible readings in the colorimeter were not possible.

The monoamino-acids

The monoamino-acids in depocasein were investigated by the method of Dakin [1918] using 440 g. = 394 g. of dry ash-free protein. After hydrolysis with 21 % HCl for 19 hr., at which period it was found to be complete, as tested by titration with neutralized formalin, the solution was evaporated *in vacuo* several times to remove excess of HCl and neutralized with NaOH. The precipitate of humin and tyrosine was filtered off and 17·9 g. of recrystallized tyrosine with N = 7·69 % (calc. 7·73 %) were isolated, equivalent to 4·5 % in depocasein. The concentrated solution was then extracted numerous times by simple shaking with an equal volume of butyl alcohol. This procedure was found by Plimmer & Lowndes (unpublished) to be as effective as a continuous extractor with the advantage that the extracted amino-acids are not heated for a long time with butyl alcohol thus preventing the formation of anhydrides. The butyl alcohol was distilled off *in vacuo* after each extraction and the amino-acids separated off. Shaking with butyl alcohol was continued till only very small amounts were dissolved and at this stage the extract was found to contain small quantities of diamino-acids.

The butyl alcohol extract was examined for proline and simple monoamino-acids. The solid amino-acids were boiled with successive portions of absolute alcohol until extraction was complete. The alcohol was evaporated off and the

residue thoroughly extracted with cold absolute alcohol. The residue from this solution was dissolved in hot water and allowed to cool. The precipitate, probably of peptide anhydrides, contained 1·21 g. N and was not further examined. The solution, clarified with charcoal and brought to 500 ml., contained 4·45 g. total N and 2·89 g. non-amino-N. Two methods were used to isolate derivatives of proline from this solution.

(*a*) *Proline picrate by Town's method* [*1928*]. 100 ml. were boiled with $CuCO_3$. The dried copper salts were extracted with methyl alcohol and gave 8·8 g. material, from which a solution was obtained containing 0·53 g. of total N and 0·36 g. of non-amino-N. The calculated amount of picric acid was added to 50 ml. of this solution and ultimately 1·4 g. of crystals with M.P. of 146–148°, not altered by recrystallization, resulted. This yield gives 1·14 % of proline in depocasein.

(*b*) *Proline hydantoin by Dakin's method* [*1918*]. 100 ml. of the solution were evaporated with 8 g. of KCNO (5·2 g. theoretical). The sticky residue after treatment with H_2SO_4 and ether finally yielded 1·13 g. of opaque white crystals with M.P. 156–158°. Dakin gave 165–167° as M.P. of *l*-proline hydantoin and 153–156° for the racemic compound. The crystals contained 22·2 % N (calc. 20·0 %). The high figure may be due to the presence of some polymerized cyanic acid. The yield corresponds to 1·18 % in depocasein.

Though only 1·2 % proline was isolated the content in depocasein is undoubtedly greater. The solution from the copper salt after boiling with an equal volume of conc. HCl contained 0·0675 g. non-amino-N which is equivalent to 2·8 %. Caseinogen according to Dakin contains 8 % proline.

The separation of the other monoamino-acids insoluble in absolute alcohol was carried out by the ester method. The ester hydrochlorides dissolved in absolute alcohol were treated with the calculated amount of Na in alcohol and NaCl was precipitated with ether. The solution after being shaken with, and filtered through, dry Na_2SO_4 was evaporated *in vacuo*, the alcoholic distillate being collected in dilute HCl to trap low-boiling esters. The esters were then fractionally distilled *in vacuo* using an oil bath and a three-stage mercury diffusion pump, kindly placed at our disposal by Dr Barrow at Birkbeck College. Between the receiver and the pump a trap was inserted immersed in a mixture of solid CO_2 and alcohol to condense vapours escaping the condenser. The following fractions were obtained:

M_1 from alcohol distillate of esters.
M_2 at 58–90° oil bath temp. 60° vapour T. 3·0 mm. Hg pressure.
M_3 from trap in freezing mixture.
M_4 at 90–100° oil bath T. 65° vapour T. 1·5 mm. Hg pressure.
M_5 at 100–120° oil bath T. 0·7 mm. Hg pressure.
No distillate 120–140° oil bath T.
M_6 at 140–180° oil bath T.
M_7 from trap in freezing mixture.

Fraction M_1 was evaporated to dryness and the residue dissolved in water, neutralized and fractionally crystallized. 3·4 g. of crystals with 11·5 % N were obtained and treated by the copper salt method (below). The remainder of the residue was NaCl.

Fractions M_2, M_3, M_4, M_5 were separately boiled with water under a reflux for 15 hr. Except M_5, which was slightly alkaline and was previously extracted with ether to remove any phenylalanine or serine ester, the solutions were neutral. The amino-acids in these fractions were submitted to extensive frac-

tional crystallization from water and alcohol. Leucine was obtained from each fraction. The final portion had a N content of 10·65 % (calc. 10·69 %). When no further separation of leucine could be obtained, the remainders and fraction M_1 were mixed, dissolved in water and converted into copper salts as described by Brazier [1930]. The copper salts insoluble in cold water gave leucine with 10·64 % N; those soluble in water were separated into copper salts soluble and insoluble in methyl alcohol. Both fractions were found to contain only valine with 11·80 % N (calc. 11·97 %) and leucine with 10·60 % N. The total pure leucine isolated amounted to 38·5 g. The content of leucine in depocasein would thus be 9·8 %. The valine, amounting to 7·9 g., had a rotation of $[\alpha]_D = 20°$ and would consist of 25 % valine, equal to 0·3 % in depocasein. The remainder corresponds to 1·7 % leucine, making leucine at least 11·5 % of the protein.

Fractions M_6 and M_7 were combined, mixed with water and extracted with ether. The ether-soluble part of M_5 was added. The ether extracts were shaken with conc. HCl and gave on hydrolysis, evaporation and crystallization 2·8 g. of phenylalanine hydrochloride with 17·8 % Cl (calc. 17·6 %). 2·1 g. of phenylalanine were prepared from it by solution in water, neutralization with ammonia and treatment with alcohol. The crystals contained 8·92 % N (calc. 8·48 % N). The high value is probably due to the presence of a little NH_4Cl. The yield is equivalent to 0·6 % in depocasein. The aqueous part was hydrolysed with baryta and examined for dicarboxylic acids and serine. Derivatives of these acids were not isolated, though there was some barium salt insoluble in alcohol and some very soluble amino-acid. Alanine was not found in any of the first fractions of esters. From the large amount of N remaining unextracted by butyl alcohol it is probable that it remained in the aqueous solution.

The residual aqueous solution, after removal of butyl alcohol by evaporation *in vacuo*, was brought to 1 l. and contained 29 g. N. An analysis by Van Slyke's method on 15 ml. without further hydrolysis, gave the following distribution of N:

	% N	Calc. to original depocasein
Amide	4·3	—
Humin	0·7	—
Total N of bases	35·2	19·4
Arginine	14·1	7·8
Histidine	0·3	0·2
Lysine	20·8	11·4
Total monoamino	56·3	31·0
Amino	50·7	28·0
Non-amino	5·6	3·0

The second column, obtained by multiplying the first by 29·0/52·7, shows the distribution as a fraction of the total N of the depocasein taken for hydrolysis. Comparison with the figures for depocasein shows no great difference in arginine; histidine has been reduced and lysine is slightly higher. It is possible that histidine, which is less basic than the two other bases, has been dissolved by butyl alcohol. The monoamino filtrate contains nearly half the total monoamino-acids of the depocasein despite the numerous extractions with butyl alcohol until only very small amounts were removed. Dakin [1920] found that the extraction of the monoamino-acids from gelatin was by no means complete, also observed by Plimmer & Lowndes (unpublished), whether the extraction be made with a continuous extractor or by frequent shaking with fresh portions of butyl alcohol. Dakin's description of the extraction of the amino-acids from hydrolysed caseinogen leads to the conclusion that it is complete. The non-amino-N of the monoamino solution amounting to 3 % on the original depocasein may be due to

the presence of peptide anhydrides or hydroxyproline. Proline was extracted by the butyl alcohol.

The solution should contain only the basic amino-acids and the dicarboxylic acids. It was used only for isolation of the latter. Two methods for the isolation of glutamic and aspartic acids were tried.

(1) *Glutamic acid as hydrochloride.* 500 ml. of the aqueous solution were evaporated and saturated with dry HCl gas. The crystalline precipitate consisted almost entirely of NaCl. It was not possible to obtain the insoluble zinc glutamate. The solution of the precipitate contained only 0·08 g. N.

(2) *Barium aspartate and barium glutamate.* 150 ml. of the solution were treated with cryst. $Ba(OH)_2$ and precipitated with alcohol according to Jones & Moeller [1928]. From the insoluble barium salts the insoluble lead salt of aspartic acid was made. The solution of the lead salt, freed from lead, contained N equivalent to 0·14 *M* aspartic acid. The preparation of the copper salt by adding 1·5 *M* copper acetate was not successful though the solution contained 0·157 g. N equivalent to 1·4 g. aspartic acid or 2·5 % of the depocasein.

The filtrate from the insoluble lead aspartate would not yield glutamic acid hydrochloride. Hydroxyglutamic acid was also not found. It is not possible to explain the non-isolation of these dicarboxylic acids, especially glutamic acid.

Further examination for dicarboxylic acids

As these acids could not be isolated from the solution remaining after butyl alcohol extraction a further hydrolysis of depocasein with HCl was carried out with approx. 40 g. material. The hydrolysed solution was evaporated *in vacuo* and found to contain 4·5 g. N = 33·8 g. depocasein.

The basic amino-acids were removed from the solution by precipitation with a slight excess of phosphotungstic acid. The filtrate and washings were evaporated *in vacuo* and the concentrated solution saturated with dry HCl gas. The precipitate, which contained glutamic acid hydrochloride and inorganic matter was first recrystallized as hydrochloride; Cl was removed with Ag_2SO_4, SO_4 and NH_4 ions with $Ba(OH)_2$ and after quantitatively removing Ba ions there were isolated 1·2 g. glutamic acid with 9·4 % N (calc. 9·5 % N) which gives a content of 3·6 % in depocasein.

The filtrate from glutamic acid hydrochloride was evaporated *in vacuo* to remove HCl and the residue dissolved in water and treated with ammonium rhodanilate according to Bergmann [1935]. From the rhodanilate 0·4 g. of nearly pure proline with 11·8 % N (calc. 12·2 % N) was isolated which corresponds with 1·2 % in depocasein, comparable with the figure obtained from the butyl alcohol extract. The quantity in depocasein is, however, much larger as the whole of the proline was not crystallized out. The filtrate from proline rhodanilate was treated with pyridine, and the solution precipitated with ammonium reineckate. The reineckate did not give any hydroxyproline in a crystalline state.

Excess reineckate was removed from the solution by means of pyridine, made alkaline with NH_4OH and $BaCl_2$ added till there was no further precipitation. The filtrate on evaporation *in vacuo* gave 2·0 and then 0·4 g. impure tyrosine with 7·42 % N. It was then treated with solid $Ba(OH)_2$ according to Jones & Moeller [1928] and poured into 5 vol. of 95 % alcohol. The insoluble Ba salts were filtered off, washed, redissolved and reprecipitated with alcohol. From the insoluble Ba salts there were ultimately obtained 3·4 g. copper aspartate, from which 1·0 g. of pure aspartic acid with 10·25 % N (calc. 10·5 % N) equivalent to 3·0 % in depocasein was isolated. Glutamic acid could not be found in the insoluble Ba salts. The alcoholic filtrates from the insoluble Ba salts were

evaporated *in vacuo*, acidified with H_2SO_4, filtered from $BaSO_4$, Cl being removed with Ag_2SO_4, Ag with H_2S and finally SO_4 quantitatively with $Ba(OH)_2$. On evaporation the solution gave 0·4 g. tyrosine. Together with the previous amounts the total tyrosine obtained by crystallization was 2·8 g. Pure tyrosine weighing 1·8 g. with 7·71 % N (calc. 7·73 % N) was prepared from this by recrystallization. The yield corresponds with 5·4 % in depocasein. Further evaporation gave firstly 2·7 g. impure leucine from which 2·1 g. with 10·6 % N was obtained on recrystallization equivalent to 6·3 % of depocasein, and then fractions with 11·2, 12·0, 11·35 % N and finally a fraction of 0·05 g. with 12·84 % N, which indicated that alanine or other lower amino-acids were present in depocasein. The experiment, essentially for the isolation of the dicarboxylic acids, was also a preliminary trial to isolate amino-acids in sequence from the same hydrolysate.

Summary

The substance produced from caseinogen by treatment with 1 % NaOH at 37° for 24 hr. and subsequent acidification is not simply a residue of caseinogen from which only phosphorus and amide groups have been removed, but a product containing 55 % of the original N of the protein. The caseinogen is apparently split into two almost equal halves. This half is preferably called depocasein, as it resembles a metaprotein in solubility in 0·1*N* acid and alkali, but is precipitated by a higher concentration of acid. Its composition in amino-acids is generally very similar to that of caseinogen, but there are a few marked differences:

	Depocasein	Caseinogen
Glycine	0·2	0·5
Alanine	?	1·5
Valine	0·3	7·2
Leucine	11·5	9·4
Phenylalanine	0·6	3·2
Aspartic acid	3·0	4·1
Glutamic acid	3·6	21·0
Proline	1·5	8·0
Tyrosine	7·7	6·4
Tryptophan	1·3	1·4
Cystine	0·3	0·4
Methionine	2·1	2·9
Histidine	1·6	1·8
Arginine	3·3	3·9
Lysine	4·5	6·3

The differences from caseinogen are in tyrosine which is over 1 % higher, methionine which is 0·8 % lower and lysine which is lower; the figure of 4·5 % is, however, too low. Proline is lower, but is probably at least 3 %. There is a difference in aspartic acid but our figure is probably too low. The most striking difference is in glutamic acid. Further analyses of depocasein are contemplated. Depocaseose is under investigation.

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LXVI. EXPERIMENTS RELATING TO THE CONSTITUTION OF ALLOXAZINE-ADENINE-DINUCLEOTIDE

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It was shown by Das [1936; 1938] that the *d*-amino-acid oxidase of Krebs [1933] was associated with a coenzyme. The latter substance was found by Straub [1938] to contain flavin and later isolated in a pure condition by Warburg & Christian [1938, 1] and reported to be an alloxazine-adenine-dinucleotide.

This coenzyme is becoming increasingly important. Warburg & Christian stated that in addition to its combination with the apoenzyme of *d*-amino-acid oxidase it formed, with other specific proteins, xanthine oxidase and enzymes capable of transferring hydrogen from dihydropyridine nucleotide to oxygen and methylene blue respectively. It is also the prosthetic group of a flavo-protein, isolated from heart muscle by Straub [1939], which was later reported by Straub *et al.* [1939] to be identical with diaphorase (coenzyme factor).

The constitution given to the substance by Warburg & Christian was based on its elementary composition and the liberation of one molecule of adenine by acid hydrolysis and one molecule of lumiflavin by alkaline photolysis. The suggestion was later made that the "old" yellow enzyme was not a natural product but resulted in the course of preparation by the fission of adenylic acid from the dinucleotide [Warburg & Christian, 1938, 2].

The present work was concerned with splitting the substance by mild acid and alkaline hydrolysis and investigating the products formed by enzymic reactions. In connexion with the adenine-containing part of the molecule use was made of the fact that the adenosine-5-phosphoric acids, which are coenzymes of the phosphorylase described by Euler *et al.* [1936, 1, 2], act as activators in the enzymic dephosphorylation of phosphoglyceric acid. The appearance of co-phosphorylase on hydrolysis showed the presence in the molecule of linkages between adenine, carbohydrate and phosphoric acid which are those of muscle adenylic acid. The flavin part of the molecule was investigated, after hydrolysis, in the presence of the protein component of the "old" yellow enzyme, using Warburg's system for the oxidation of Robison ester. In this system a considerable proportion of the original activity of the dinucleotide remained after treatment which completely destroyed its ability to oxidase *d*-alanine. This indicated the production, on hydrolysis, of lactoflavin-5-phosphoric acid and the presence of this residue in the dinucleotide.

Experimental

Alloxazine-adenine-dinucleotide

The coenzyme was prepared as its Ba salt (24 mg. from 10 kg. yeast) by the method of Warburg & Christian [1938, 1], with whose product it appeared to be identical. The phosphorus content, determined colorimetrically, was 6·5%

($C_{27}H_{31}O_{15}N_9P_2Ba$ requires P 6·74 %). Spectrophotometric measurements were kindly carried out by Mr G. Günther. At $\lambda = 3650$ a $2{\cdot}8 \times 10^{-5} M$ solution by weight was found to contain $2{\cdot}7 \times 10^{-5}$ mols per litre, on the basis of the curve given by Warburg & Christian.

Phosphorylase system

The reaction measured was the dephosphorylation of phosphoglyceric acid. The enzyme preparation was obtained by acetone precipitation from rat muscle extract. Minced rat muscle was extracted twice with twice its weight of water, the extract dialysed 18 hr. against 0·9 % KCl, precipitated with 10 vol. acetone at $-8°$, and the precipitate washed with absolute alcohol and dried. For experiment 0·25 g. of the precipitate was extracted with a mixture of 1 ml. 0·04 *M* $MgSO_4$, 4 ml. of water and 6 ml. of veronal buffer *p*H 7·4, and the insoluble material separated by centrifuging.

In each determination were used 0·8 ml. of enzyme solution, 0·5 ml. of phosphoglyceric acid (15 mg. of Ba salt per ml. decomposed with *M*/10 Na_2SO_4) and 1·2 ml. of water containing the activator to be investigated. After incubation for 2 hr. at 30° 3 ml. of 10 % trichloroacetic acid was added and the protein removed by centrifuging. Free phosphoric acid was determined in 1 ml. of the resulting solution by the method of Teorell [1931].

Protein component of the "old" yellow enzyme

Crude yellow enzyme from yeast was purified by adsorption on alumina *C*-γ using the method of Weygand & Stocker [1937]. The prosthetic group was then separated by the procedure of Warburg & Christian [1938, 2]. 4 ml. of a solution containing $0{\cdot}2 \times 10^{-6}$ mol. of flavin enzyme were mixed with 4 ml. of saturated ammonium sulphate and cooled to 0°. 0·1 *N* HCl was then added slowly with shaking until the *p*H of the solution was approximately 2·8. The precipitated protein was washed at 0° at the centrifuge with 7 ml. of half-saturated ammonium sulphate, dissolved at 0° in 1 ml. of *M*/2 phosphate buffer, *p*H 7·4 and diluted to 10 ml. The flavin solution was neutralized with *M*/2 Na_2HPO_4 and also diluted to 10 ml. (denoted by F.M. in Table III).

Protein component of d-*amino-acid oxidase*

From an extract of acetone-dried sheep's kidney [Krebs, 1935] a solid preparation containing the required protein was obtained by the method of Warburg & Christian [1938, 1], separation of the prosthetic group being effected in 35 % saturated ammonium sulphate solution at *p*H 2·8. 100 ml. of kidney extract yielded 150 mg. of material.

Oxidation of Robison ester and d-*alanine*

After hydrolysis by *N*/20 HCl and NaOH the activity of the coenzyme, in combination with the specific proteins, was tested in two different systems, the oxidation of *d*-alanine and of Robison ester.

The experiments were carried out in Warburg vessels at 30°. In each case the gas used was pure O_2 and the centre cups contained 0·2 ml. of 5 % KOH. In the Robison ester system there was used in each vessel $5{\cdot}0 \times 10^{-9}$ mol. of the co-enzyme (denoted by F. A. D.) and protein corresponding to that obtained by

splitting the same amount of "old" yellow ferment. For the oxidation of alanine the vessels contained, in the case of hydrolysis with HCl, 10 μg. of the coenzyme and 3 mg. of the protein preparation; the coenzyme was in large excess, parallel experiments showing that the rate of O_2 uptake using 2 μg. was approximately only 5% less. In the case of hydrolysis with NaOH there was used 0·5 μg. of the co-enzyme with 4 mg. of protein.

Results

Production of cophosphorylase from the dinucleotide

Cophosphorylase experiments were carried out, in the system described, on the intact coenzyme and after hydrolysis of the latter under mild alkaline and acid conditions. Table I summarizes the results obtained.

Table I

Activator	μg.-atom P per ml.	% dephosphorylation
0·0125 mg. adenylic acid	1·66	20·2
0·025 "	2·70	32·7
0·050 "	3·29	39·9
0·10 "	3·95	47·9
0·20 "	4·44	53·8
0·025 mg. adenosine triphosphoric acid	2·00	24·2
0·10 " "	3·13	37·9
0·40 " "	4·45	53·8
0·636 mg. coenzyme in water	1·84	22·3
0·636 mg. coenzyme after treatment with *N*/20 NaOH 15 min. at 18° and neutralization with *N*/20 HCl	3·55	43·1
0·636 mg. coenzyme treated with *N*/20 NaOH 10 min. at 18° and 5 min. at 95°. Neutralized with *N*/20 HCl	3·88	47·1
0·636 mg. coenzyme treated with *N*/20 HCl 10 min. at 95° and neutralized with *N*/20 NaOH	3·22	39·0
No activator	1·16	14·1

By comparison of the amounts of dephosphorylation when adenylic acid and the coenzyme were used as activators an estimate can be made of the amount of splitting of the latter, in terms of the possible production of adenosine-5-phosphoric acid, under the conditions employed (Table II).

Table II

Treatment	% of theoretical splitting
Coenzyme in water	<5
Coenzyme in *N*/20 NaOH 15 min. at 18°	28
Coenzyme in *N*/20 NaOH 10 min. at 18° and 5 min. at 95°	40
Coenzyme in *N*/20 HCl 10 min. at 95°	20

Effect of hydrolysis of the coenzyme on its activity in combination with the protein components of d-*amino-acid oxidase and the "old" yellow enzyme*

(1) *Hydrolysis with acid.* The results are shown in Table III. In exps. (*a*) and (*b*) different samples of Robison ester and "Zwischenferment" were used in the one system and a fresh protein preparation in the other.

In the oxidation of Robison ester the activity of the coenzyme, after treatment with $N/20$ HCl, was very similar whether the time of hydrolysis was 30 or 120 min., the rate of O_2 uptake, after subtraction of the blank values, being about 70 % of that with the unchanged substance. Under the same conditions the coenzyme activity with respect to the oxidation of *d*-alanine was completely destroyed.

Table III

(1) Oxidation of Robison ester

Protein solution $M/2$ phosphate *p*H 7·4 Zwischenferment Robison ester (0·1 m.) $N/20$ KCN	0·25 ml. 0·2 ml. 0·5 ml. 0·4 ml. 0·2 ml.	⟶	⟶	⟶	⟶
H_2O	0·45 ml.	/	/	/	0·2 ml.
Coenzyme	/	0·45 ml. F.A.D.	0·45 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	0·45 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	0·25 ml. "split" F.M.
Co_{II} (in side cup)	0·2 ml. (0·05 mg.)	⟶	⟶	⟶	⟶
Time (min.)	µl. O_2	µl. O_2	µl. O_2	µl. O_2	µl. O_2
(*a*) 10	8·8	41·9	32·3	32·8	37·6
20	14·0	67·6	51·0	54·1	61·6
30	21·1	91·8	70·5	75·4	83·8
40	25·5	114·3	88·4	94·3	102·6
(*b*) 10	7·5	45·9	34·0	30·6	—

(2) Oxidation of *dl*-alanine

Protein solution	3 mg. in 1 ml. $N/10$ pyrophosphate *p*H 8·3	⟶	⟶	⟶
H_2O	/	/	/	1·0 ml.
Coenzyme	1·0 ml. F.A.D.	1·0 ml. F.A.D. (30 min. in $N/20$ HCl at 95°)	1·0 ml. F.A.D. (120 min. in $N/20$ HCl at 95°)	/
4·5 % alanine (in side cup)	0·2 ml.	⟶	⟶	⟶
Time (min.)	µl. O_2	µl. O_2	µl. O_2	µl. O_2
(*a*) 10	11·3	0	0	0
20	24·1	0	0	0
30	35·4	0	0	0
40	46·7	0	0	0
(*b*) 10	14·2	0	0	0

(2) *Hydrolysis with alkali.* Treatment of the coenzyme with $N/20$ NaOH was carried out at room temperature, as heating at 95° brought about rapid disappearance of the flavine colour. But even under these conditions it did not appear possible to destroy selectively, as in the case of acid hydrolysis, the activity with respect to the oxidation of *d*-alanine. Table IV gives the values for the O_2 uptake. Under the conditions employed, hydrolysis for 15 and 60 min. reduced the rate of O_2 uptake in the Robison ester system to about 75 % and 45 % respectively of that obtained using the untreated coenzyme. In the oxidation of alanine the corresponding values were about 93 and 58 %.

Table IV

(1) Oxidation of Robison ester

Protein solution	0·25 ml.			
$M/2$ phosphate pH 7·4	0·2 ml.			
Zwischenferment	0·5 ml.	→	→	→
Robison ester (0·1 M)	0·4 ml.			
$N/20$ KCN	0·2 ml.			
H_2O	0·45 ml.	/	/	/
Coenzyme	/	0·45 ml. F.A.D.	0·45 ml. F.A.D. (15 min. in $N/20$ NaOH at 18°)	0·45 ml. F.A.D. (60 min. in $N/20$ NaOH at 18°)
Co_{II} (in side cup)	0·2 ml. (0·05 mg.)	→	→	→
Time (min.)	μl. O_2	μl. O_2	μl. O_2	μl. O_2
10	10·6	45·3	37·8	26·2
20	16·7	71·8	59·6	41·0
30	21·2	94·0	78·9	54·1
40	23·8	112·9	91·8	64·0

(2) Oxidation of *dl*-alanine

Protein solution	4 mg. in 1 ml. $N/10$ pyrophosphate pH 8·3	→	→	→
H_2O	/	/	/	1·0 ml.
Co-enzyme	1·0 ml. F.A.D.	1·0 ml. F.A.D. (15 min. in $N/20$ NaOH at 18°)	1·0 ml. F.A.D. (60 min. in $N/20$ NaOH at 18°)	/
4·5% alanine (in side cup)	0·2 ml.	→	→	→
Time (min.)	μl. O_2	μl. O_2	μl. O_2	μl. O_2
10	11·7	10·5	6·3	0
20	22·5	20·9	12·7	0
30	33·4	32·2	19·9	0
40	44·3	41·9	27·1	0

After treatment of the coenzyme with $N/20$ HCl at 95 for 30 min. or with $N/20$ NaOH at 18° for 60 min. no visible yellow colour was extracted from the resulting solutions, at pH 5, by benzyl alcohol. As this solvent has been shown by Emmerie [1938] to extract flavin, though not flavin phosphoric acid, it appeared that significant amounts of the former substance were not produced under these conditions.

Conclusions

The experiments on the production of cophosphorylase show that adenosine-5-monophosphoric acid is a constituent of the new dinucleotide and is involved with a linkage which is easily hydrolysed, especially under alkaline conditions. The very small amount of cophosphorylase present after using the untreated substance is possibly due to enzymic splitting in the system employed.

The formation of adenosine-5-phosphoric acid, in conjunction with the results obtained in the Robison ester and alanine systems, indicates that in the first stage of acid hydrolysis a considerable proportion of the coenzyme breaks down into two mononucleotides, the reaction which was suggested by Warburg & Christian [1938, 2] to occur enzymically in Lebedew-Saft. The fact that little difference was found in the activity of the coenzyme, with respect to the oxidation of Robison ester, whether treated with $N/20$ HCl for 30 or 120 min. is explained by the slow rate of hydrolysis of lactoflavin-5′-monophosphoric

acid in acid solution [Kuhn & Rudy, 1936]. The difficulty of destroying selectively, in NaOH solution, the activity of the coenzyme as the prosthetic group of *d*-alanine oxidase, may be assigned to the lability of lactoflavin towards alkali.

Summary

1. The coenzyme of *d*-amino-acid oxidase has been prepared by the method of Warburg & Christian with whose product it was identical in phosphorus and flavin contents.

2. Hydrolysis of the coenzyme with dilute acid and alkali lead to the production of cophosphorylase, showing the presence in the molecule of adenosine-5-monophosphoric acid.

3. By acid hydrolysis it was possible to destroy the activity of the coenzyme with regard to the oxidation of *d*-alanine while maintaining, to a considerable extent, its ability to transfer hydrogen from dihydropyridine nucleotide. This indicated the liberation, by hydrolysis, of lactoflavin-5-phosphoric acid.

4. It has been suggested that the coenzyme can be split enzymically into two mononucleotides, adenylic acid and lactoflavin-5-phosphoric acid [Warburg & Christian, 1938, 2]. It now appears that this reaction can occur on acid hydrolysis.

I wish to thank Prof. H. von Euler for the facilities he has provided and his interest in this work, and Dr F. Schlenk for advice and criticism.

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LXVII. COENZYME-LINKED REACTIONS INVOLVING *l*(+)GLUTAMIC DEHYDROGENASE

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l(+)Glutamate in the presence of its dehydrogenase reduces coenzyme I [Dewan, 1938; Euler *et al.* 1938]. Reduced coenzyme in turn is oxidized enzymically by substrates such as aldehyde, acetoacetate, pyruvate and oxaloacetate [Green & Dewan, 1937; Euler *et al.* 1936; 1937]. The possibility of linkages between glutamate and these systems via coenzyme I would therefore seem likely. Adler *et al.* [1937] have linked the glutamate and alcohol dehydrogenase systems, and Dewan [1938] the glutamate and β-hydroxybutyrate systems.

This present communication describes coenzyme-linked reactions between the *l*(+)glutamic dehydrogenase system on the one hand and the malic and lactic systems on the other.

I. Preparation of the dehydrogenases

A very active preparation of glutamic, malic and lactic dehydrogenases may be obtained from pig heart muscle in the following manner. Two pig hearts are divested of fat and connective tissue, minced in a Latapie, washed 5 times with tap water and ground in a mortar with sand and 250 ml. *M*/25 phosphate buffer *p*H 7·2 for 1½ hr. The thick homogeneous paste is mixed with 350 ml. of the same buffer and centrifuged 5 min. to remove sand and cellular debris. The supernatant is brought to *p*H 4·6 (slightly yellow to chlorophenol red) with 10% acetic acid and centrifuged for 10 min. The precipitate is rubbed up with distilled water and again centrifuged 10 min. It is finally suspended in 70 ml. *M*/5 phosphate buffer *p*H 7·2. The preparation keeps its activity for about 5 days.

II. Methods of estimation

Since oxidation of *l*(+)glutamate results in the production of NH_3 and α-ketoglutarate [Euler *et al.* 1938; Dewan, 1938] the oxidation can be followed by estimation of NH_3 (Parnas method).

Lactic and malic acids were estimated by the manometric method described by Dewan & Green [1937].

III. Reaction between glutamate and pyruvate

The reactions were carried out anaerobically in Thunberg tubes at 38° for 1½ hr. Table I shows that glutamate is oxidized by pyruvate only in the presence of the enzymes and coenzyme. Lactic acid was also found only when the whole system was present. Table II shows that the lactic acid found was in close agreement with the theoretical amount calculated on the assumption that 1 mol. of lactic is formed for each mol. of NH_3 produced.

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Table I. *Controls for glutamate-pyruvate oxidoreduction*

Heart enzyme (ml.)	2·0	2·0	2·0	2·0	—
0·3% coenzyme I (ml.)	1·0	1·0	1·0	—	1·0
M/3 *l*(+)glutamate (ml.)	0·5	0·5	—	0·5	0·5
M pyruvate (ml.)	0·3	—	0·3	0·3	0·3
Water (ml.)	—	0·3	0·5	1·0	2·0
NH_3 (mg.)	0·15	0·01	0	0	0

Table II. *Ratio of observed to theoretical lactic acid calculated from NH_3 produced*

	Lactic acid in μl. O_2	Theory	Ratio
(1)	60	66	0·96
(2)	82	78	1·1
(3)	98	106	0·93

Table III. *Controls for the glutamate-oxaloacetate oxidoreduction*

Heart enzyme (ml.)	2·0	2·0	2·0	2·0	—
0·3% coenzyme I (ml.)	1·0	1·0	1·0	—	1·0
M/3 *l*(+)glutamate (ml.)	0·5	0·5	—	0·5	0·5
M/3 oxaloacetate (ml.)	0·3	—	0·3	0·3	0·3
Water (ml.)	—	0·3	0·5	1·0	2·0
NH_3 (mg.)	0·18	0·02	0	0	0

Table IV. *Ratio of observed to theoretical malic acid*

	Malic acid in μl. O_2	Theory	Ratio
(1)	122	114	1·1
(2)	86	72	1·1
(3)	88	102	0·9

IV. Reaction between glutamate and oxaloacetate

Oxaloacetate produces similar results when used as oxidizing agent (cf. Table III). Malic acid was also found only when the complete system was present. Table IV shows that the malic acid produced was approximately equal to the theoretical amount calculated on the assumption that 1 mol. is produced for each mol. of NH_3.

Oxaloacetic acid tends to decompose at 38° to pyruvic acid and CO_2. There was therefore the possibility of pyruvic acid acting as oxidizing agent. This was not so, however, since lactic acid was not formed under the conditions of the experiment.

Summary

The anaerobic oxidation of *l*(+)glutamate by pyruvate and oxaloacetate via coenzyme I has been described.

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LXVIII. MICRODETERMINATION OF GLUTAMIC ACID

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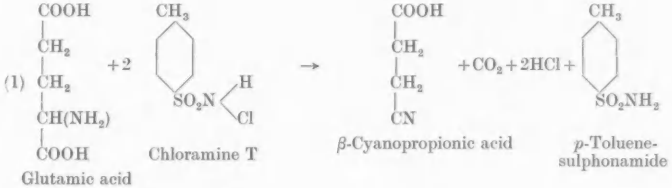
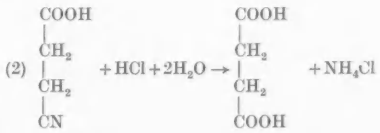
(*Received 1 March 1939*)

In the course of experiments on the intermediary metabolism of amino-acids it was found necessary to determine small quantities of glutamic acid. Methods used by previous workers either require large quantities or are unspecific [Foreman, 1914; Jones & Moeller, 1928; Braunstein & Kritzmann, 1937]; on the suggestion of Dr H. A. Krebs a new method has therefore been worked out which is more specific than the older methods and permits the accurate determination of glutamic acid in amounts from 0·1 mg. upwards.

Principle of the method

Glutamic acid is converted into succinic acid by treatment with an excess of chloramine T and subsequent acid hydrolysis [Dakin, 1917]:





The succinic acid is determined manometrically [Szent-Györgyi & Göszy, 1935; Krebs, 1937].

Optimum conditions for reaction (2)

β-Cyanopropionic acid was prepared from glutamic acid according to Dakin [1917], and the optimum conditions for its hydrolysis determined by studying the rate of succinic acid formation.

Reaction (2) occurs readily in the presence of strong HCl at 100°. The influence of HCl concentration on the reaction is given in Table I. Hydrolysis is complete within 15 min. when the concentration is at least 12·5%. The effect of the time of heating on the rate of hydrolysis is given in Table II. Since prolonged heating is to be avoided (see discussion of specificity) heating for 15 min. with 12·5% HCl was adopted as the standard procedure.

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Table I. *Influence of HCl concentration on hydrolysis of β-cyanopropionic acid*

Heating time 15 min., 100°. After hydrolysis succinic acid was extracted and determined manometrically.

	mg. β-Cyano-propionic acid	% HCl (final conc.)	Succinic acid found (μl. O_2)	Succinic acid calc. (μl. O_2)	Hydrolysis %
1	3·22	2·6	153	366	42
2	3·22	6·5	249	366	68
3	3·22	10·4	344	366	94
4	3·22	12·5	363	366	99
5	3·64	15·0	414	414	100

Table II. *Effect of time of heating on rate of hydrolysis of β-cyano-propionic acid*

12·5% HCl. After heating succinic acid was extracted and determined manometrically.

	mg. β-Cyano-propionic acid	Time of heating min.	Succinic acid found (μl. O_2)	Succinic acid calc. (μl. O_2)	Hydrolysis %
1	3·69	0	12·3	418·5	3
2	3·69	2·5	105·3	418·5	25
3	3·69	5	276·3	418·5	66
4	3·69	7·5	345·3	418·5	83
5	3·64	10	393·6	414	95
6	3·64	15	413·1	414	100
7	3·64	20	414·6	414	100

Optimum conditions for reaction (1)

Reaction (1) was studied by determining the formation of β-cyanopropionic acid by measuring the succinic acid formed from it on acid hydrolysis.

Dakin [1917] found that reaction (1) took place only when the molar concentration of chloramine T was at least twice that of glutamic acid. We find that an excess of chloramine T does not interfere. The investigation of the influence of time, temperature and *p*H on reaction (1) showed that quantitative yields of β-cyanopropionic acid were obtained when the reaction was carried out at 40° for 10 min. at *p*H 4·7. At *p*H 2·7 the average recovery was 83%, while at *p*H 7 the recoveries were as low as 38%. Thus it is important to keep the *p*H in the range of 4–5. At high temperatures (100°) the recoveries average 20%.

The rate of reaction (1) may be followed manometrically by measuring the CO_2 produced. From Table III it can be seen that the theoretical value for CO_2 is obtained within 5 min. It should be noted however that the final CO_2 value is slightly higher than the theoretical. This extra CO_2, which amounts to 1–2·5%,

Table III. *Rate of CO_2 production from glutamic and β-cyanopropionic acids measured manometrically*

Each cup contains 2 ml. 10% chloramine T, 1·1 ml. citrate buffer, *p*H 4·7, 2 ml. substrate solution. Temp. 40°. 1·51 mg. glutamic acid is expected to yield 230 μl. CO_2 according to (1).

Substrate	Time min.	μl. CO_2 formed	Yield of CO_2 % of theory
Glutamic acid (1·51 mg.)	5	231·4	100·5
	10	233·1	101·2
	15	235·1	102
	20	235	102
β-Cyanopropionic acid (1·62 mg.)	5	1·9	—
	10	1·7	—
	15	1·7	—
	20	2·4	—

implies that glutamic acid is oxidized by chloramine T at some other point in the molecule besides the amino group. Since this "side reaction" occurs simultaneously with reaction (1) it was not found possible to prevent it. As a result of this the corresponding yields of β-cyanopropionic acid are low by 1–2·5 %. The extra CO_2 is not due to further oxidation of β-cyanopropionic acid (Table III).

Reagents

(1) Citrate buffer (*p*H 4·7): 17·65 g. $Na_3C_6H_5O_7$, $2H_2O$ and 8·40 g. $C_6H_8O_7$, $1H_2O$ are dissolved in water and diluted to 50 ml.

(2) 0·1 *M* phosphate buffer (*p*H 7·4): 17·8 g. Na_2HPO_4, $2H_2O$ are dissolved in about 500 ml. water and 20 ml. *N* HCl added. The solution is then diluted to 1 l.

(3) 10 % chloramine T solution (to be freshly prepared before use).

(4) Conc. HCl.

(5) 5 % NH_4Cl solution.

(6) Sat. NaOH solution (50–60 %).

(7) Succinoxidase preparation.

The preparation of this enzyme system has been previously described by Krebs [1937]. As noted by Krebs, the enzyme preparation acquires an increasing blank O_2 uptake on storage. To avoid this, the following procedure may be adopted.

The finely minced pigeon muscle (about 50 g.) is washed with 500 ml. distilled water and filtered by suction through muslin. This procedure is twice repeated. The muscle pulp is sucked as dry as possible after the third washing. For use, a portion of the pulp is suspended in four to five times its weight of 0·1 *M* phosphate buffer, *p*H 7·4. For storage, it is moistened with distilled water and kept in the refrigerator in a tightly glass-stoppered bottle. Such a preparation remains active without increasing its blank O_2 uptake for as long as 7–10 days.

The fresh enzyme, prepared by suspension in 0·1 *M* phosphate buffer, usually has a small blank of 1–2 μl. O_2 per ml. suspension. If such a suspension is allowed to stand in the refrigerator for 4–5 days the blank may increase to as much as 10–15 μl. O_2 per ml. However, when the enzyme is stored as described above and freshly suspended in 0·1 *M* phosphate buffer as needed the blank is rarely greater than 2–3 μl. O_2 per ml. after as long as 1 week.

Procedure

(1) *Deproteinization.* When tissue slices are employed, it is not necessary to deproteinize, since the small amount of protein present does not interfere. Suspensions of minced tissue are deproteinized by the addition of $\frac{2}{3}$ vol. of $\frac{2}{3}N$ H_2SO_4 plus $\frac{1}{3}$ vol. of 10 % Na_2WO_4. An aliquot of the filtrate is used for the determination.

(2) *Oxidation by chloramine T.* The solution to be analysed is brought to *p*H 4·7 by the addition of 1–1·5 ml. citrate buffer. 2 ml. 10 % chloramine T are added and the solutions well mixed by shaking. They are then placed in a rack and shaken at 40° for 10 min. The reaction is carried out in small Erlenmeyer flasks, or, where tissue slices are employed, the reaction may be carried out in the manometric flask after the removal of the slices and the alkali in the centre well. After 10 min. shaking the containers are removed and placed in an ice bath for 15–20 min. to precipitate most of the *p*-toluenesulphonamide formed as a reaction product, and most of the unused chloramine T. The solutions are filtered, the precipitate is washed with several small volumes of water and the combined filtrate and washings are collected in large test tubes (25 × 200 mm.).

(2A). *Extraction of β-cyanopropionic acid.* In the determination of glutamic acid in blood, tissue extracts and other fluids containing glutathione, it is necessary to separate the β-cyanopropionic acid from another compound derived from glutathione which forms succinic acid on hydrolysis (see discussion on specificity). This is accomplished by extraction with ethyl ether in which the β-cyanopropionic acid is readily soluble, while the glutathione derivative remains in the aqueous layer.

The filtrate described above is acidified with 4 ml. 10 % H_2SO_4 and extracted in a continuous extractor of the Kutscher-Steudel [1903] type. With the extractors and conditions used in this study extraction for 1–2 hr. was usually sufficient. 2·5 ml. phosphate buffer are added to the extraction flask and the ether is slowly distilled off. The remaining aqueous solution is then chilled to precipitate the *p*-toluenesulphonamide, which is filtered off, and the filtrate containing the β-cyanopropionic acid is collected in test tubes.

(3) *Hydrolysis of β-cyanopropionic acid.* Conc. HCl is added to the filtrate to make a final concentration of not less than 12·5 %. The tubes are placed in a boiling water bath for 15 min. At the end of this time the tubes are removed and allowed to cool. Conc. NaOH (50–60 %) is added dropwise until the solution becomes hot. At this point 0·5 ml. 5 % NH_4Cl is added and the contents are well mixed. The NH_4Cl decomposes traces of chloramine T, which if present will decolorize the indicator. A few drops of phenol red are now added and the solution is made alkaline to a purple colour. A large excess of alkali should be avoided as the *p*-toluenesulphonamide forms a salt in strongly alkaline solution. The solution is then cooled and transferred to a Kutscher-Steudel extractor.

(4) *Extraction with ether.* The ether must be free from peroxides, otherwise the manometric readings will be inaccurate owing to the decomposition of the peroxides by the catalase in the enzyme preparation [Krebs, 1937]. The alkaline solution is extracted with freshly distilled ether in a Kutscher-Steudel extractor for a time sufficient to remove the remaining traces of *p*-toluenesulphonamide (usually 1–2 hr.). While *p*-toluenesulphonamide in a concentration as high as 0·10 *M* has no inhibiting effect on succinic acid oxidation, it will separate during concentration of the extract if it is not removed and thus make the quantitative transfer of the concentrated solution difficult.

After the period of alkaline extraction, the extraction flask is removed and the contents of the extractor are acidified strongly to phenol red (light yellow-pink colour) with 3 ml. 10 % H_2SO_4. A clean extraction flask with fresh ether is attached and the extraction continued for 2 or more hours, during which the phenol red is also extracted from the aqueous solution.

(5) *Preparation of solution for succinic acid determination.* When the extraction of the succinic acid is complete, 2–3 ml. *M*/10 phosphate buffer are added to the ether solution followed by dropwise addition of 2 *N* NaOH until the aqueous solution assumes the colour of neutral or slightly alkaline phenol red. The ether is distilled off and after the remaining neutral or slightly alkaline aqueous solution has been concentrated further on a steam bath to about 1 ml. it is then transferred to a small graduated cylinder by means of a small pipette. The extraction flask is rinsed with 0·5–2 ml. *M*/10 phosphate and the washings are combined with the solution.

For further details of the succinic acid determination see Krebs [1937].

Calculation

The μl. O_2 uptake observed is converted into mg. glutamic acid as follows:

$$\frac{\mu\text{l. } O_2}{112} \times 1{\cdot}47 = \text{mg. glutamic acid.}$$

Recovery of known amounts of glutamic acid

The recoveries of known amounts of glutamic acid from pure solutions are recorded in Table IV. The average recovery is 96 %. The deficit of 4 % is partly due to an over-oxidation of the glutamic acid (as evidenced by the excess CO_2 production) and partly to unavoidable loss during the various manipulations.

Table IV. *Recovery of glutamic acid*

mg. glutamic acid	% recovery	mg. glutamic acid	% recovery
0·1	99	3·02	91·5
0·36	99	3·60	97·0
0·65	100	5·27	94·4
1·05	95	6·03	98·2
1·32	96·6	8·04	95·2
2·63	92·8	10·05	91·5

The recovery values for glutamic acid added to protein solutions are of the same order as those from pure solutions. Thus, of 8·80 mg. glutamic acid added to a muscle suspension, 8·20 mg. or 94 % were recovered.

The smallest amount of glutamic acid which can be determined is limited by the accuracy of the manometric succinic acid determination. Since 1 mg. glutamic acid is equivalent to 76·2 μl. O_2, as little as 0·1 mg. can be determined by this method. Since the error in the manometric determination is greater with small readings, the method is more accurate with amounts above 1 mg.

Specificity of the method and interfering substances

Succinic acid and substances other than glutamic acid which form succinic acid during the course of the procedure will interfere with the determination unless taken into account. Among the substances tested which are likely to occur in biological material, glutamine and glutathione are the only compounds which have been found to yield significant amounts of succinic acid (see Table V).

Table V. *Succinic acid formation from compounds other than glutamic acid*

Compound	Amount (mg.)	Yield of succinic acid %
dl-Proline	29·3	0
α-Ketoglutaric acid	30·9	0
Ornithine-HCl	6·9	0
dl-Alanine	3·3	0
dl-Valine	4·4	0
dl-Leucine	2·5	0
Arginine-HCl	12·5	0
l(−)Methionine	20·0	0
l(−)Tyrosine	20·0	0
Aspartic acid	10·0	0
Asparagine	11·4	0
Oxidized glutathione	8·5	17·5
Reduced glutathione	8·5	17·5
Glutamine	1–3·6	70–80

As Weil-Malherbe [1937] has reported that α-ketoglutaric acid yields succinic acid when autoclaved with H_2SO_4, it should be noted that no succinic acid is formed from relatively large amounts of α-ketoglutaric acid under the conditions employed in this method.

Glutathione. When glutathione is treated with chloramine T under the conditions used for the determination of glutamic acid, it forms a compound or compounds, probably β-cyanopropionylcysteylglycine and the disulphide, which give rise to succinic acid on hydrolysis. In experiments where only negligible amounts of glutathione are present, the glutamic acid can be determined without separating the β-cyanopropionic acid before hydrolysis (step 2A). However, if significant amounts of glutathione are present, as in blood or in certain tissue extracts, it is necessary to remove the β-cyanopropionic acid from the interfering compounds, as described in the procedure (2 A). The recovery of glutamic acid in the presence of glutathione using step 2A is listed in Table VI.

Table VI. *Recovery of glutamic acid in presence of glutathione*

β-Cyanopropionic acid extracted before hydrolysis.

	μl. O_2 found	μl. O_2 calc.	% recovery
Glutathione (7·2 mg.)	4·9	284*	1·7
Glutamic acid (3·3 mg.)	234	251	94
Glutathione (7·2 mg.) + glutamic acid (3·3 mg.)	242	251	96

* Calc. for the equiv. quantity of glutamic acid.

Glutamine. In quantities of 1 to 3·6 mg. glutamine yields 70–80 % of the theoretical amounts of succinic acid (Table V). The yields were not improved by hydrolysis with acid or alkali under a variety of conditions before the determination. Attempts to convert glutamine into pyrrolidone-carboxylic acid under conditions described by Vickery *et al.* [1935] and subsequent conversion of the pyrrolidone-carboxylic acid into glutamic acid in acid or alkali according to Wilson & Cannan [1937] failed to improve the yields. Since no satisfactory method has been found for converting glutamine quantitatively into glutamic acid, the problem of determining glutamic acid in the presence of glutamine remains unsolved. However, since glutamine occurs only under special conditions [Krebs, 1935], this difficulty will be met with in a few instances only.

Succinic acid. When present in appreciable quantities succinic acid can be determined either separately on an aliquot [Krebs, 1937] or it can be removed from the glutamic acid solution by extraction with ethyl ether. Glutamic acid in strongly acid solution is not extracted with ether. Thus a mixture containing 2·62 mg. succinic acid plus 3·96 mg. glutamic acid was acidified with 3 ml. 10 % H_2SO_4 and extracted with ether for 30 min. The ether extract was found to contain 2·62 mg. succinic acid, or 100 % recovery, while the aqueous solution when analysed was found to contain 3·78 mg. glutamic acid or 95·5 % recovery.

Inhibitors of succinoxidase. Compounds such as malonic, fumaric, oxaloacetic and α-ketoglutaric [Weil-Malherbe, 1937] acids inhibit the oxidation of succinic acid. The last three compounds only slow up the oxidation, but do not affect the final values. For example, we found that in the presence of 0·125 *M* α-ketoglutaric acid theoretical results for the added succinic acid were obtained in 60 min.

Neither aspartic acid nor asparagine yields succinic when treated with chloramine T and subsequently hydrolysed (Table VI). However, by analogy with reactions (1) and (2) it might be expected that they would yield malonic acid. To test this, solutions of aspartic acid and asparagine were treated according to the outlined procedure. These were then added to an enzyme preparation and the O_2 uptake of a known amount of added succinic acid was determined. The theoretical malonic acid concentration in the case of aspartic acid was 0·012 *M*, of asparagine 0·014 *M*. However, the theoretical O_2 uptake of the added

succinic acid (0·0025 *M*) was observed in the same period of time as in the controls. Thus neither aspartic acid nor asparagine interferes with the determination of glutamic acid.

Application

An example showing the application of the method to studies in intermediary amino-acid metabolism is shown in Table VII in which are listed data from experiments on transamination ("Umaminierung" of Braunstein & Kritzmann [1937]). It is to be seen that the formation of glutamic acid from

Table VII. *Rate of anaerobic glutamic acid formation and disappearance in pigeon breast muscle*

1 part pigeon breast muscle suspended in 5 parts *M*/10 phosphate buffer (*p*H 7·4); final substrate concentration = 0·016 *M*.

Glutamic acid formation from α-keto-glutaric and *l*(+) alanine		Glutamic acid disappearance	
min.	mg. glutamic acid	min.	mg. glutamic acid
0	0·61	0	8·82
10	6·67	60	4·57 (pyruvic acid added)
20	5·40	60	8·20 (no pyruvic acid added)
40	5·00		

α-ketoglutaric acid and *l*(+)alanine proceeds very rapidly in minced muscle and reaches a peak within the first 10 min.; thereafter the glutamic acid formed slowly disappears. When glutamic acid alone is added to muscle it also disappears slowly, but when pyruvate is added 50 % disappears in 60 min. The findings, which are being investigated further, in general confirm those reported by Braunstein & Kritzmann [1937].

The glutamic acid contents of different tissues are listed in Table VIII. The tissues were minced shortly after the death of the animal and extracted with

Table VIII. *Glutamic acid content of tissues*

	mg. glutamic per 100 g. wet wt.
Pigeon breast muscle	34
Pigeon gizzard	94
Guinea-pig kidney	94
Guinea-pig liver	47
Sheep brain (hemispheres)	80
Sheep heart muscle	171
Blood plasma (human)	2·8

2 vol. of boiling water. The glutamic acid contents of these tissues are remarkably high. In contrast, blood plasma has a very low glutamic acid content. Determinations of the glutamic acid content of the erythrocytes from the same specimen of blood show a value of the order of 20 mg. 100 ml.

A sample of human urine was found to contain 4·3 mg. 100 ml. of glutamic acid.

Summary

A micro method for the determination of glutamic acid in biological material is described. Glutamic acid is oxidized by chloramine T to β-cyanopropionic acid; the latter is hydrolysed to succinic acid; succinic acid is determined

manometrically by means of a succinoxidase preparation. The method is highly specific; no other compound except glutamine occurring in biological material has been found to react.

The glutamic acid content of different tissues has been found to be remarkably high, the highest value observed being 171 mg. per 100 g. heart muscle.

I wish to express my thanks to Dr H. A. Krebs for suggestions and help throughout this study.

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LXIX. THE ISOLATION OF TWO TRANSFORMATION PRODUCTS OF TESTOSTERONE FROM URINE

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INCREASES in the amount of androgenic material, assayed biologically, which could be extracted from human urine following administration of androgens, have been reported by Bühler [1933], by McCullagh *et al.* [1938], who treated a eunuch with urine extract, and by Kochakian [1937]. These were small and uncertain, and were produced by low doses. Dorfman & Hamilton [1939], nsing efficient methods of extraction, have recently reported three cases in which large increases in the amount of androgenic material occurred as a result of the administration of testosterone propionate. Prof. J. W. Cook has carried out chemical work on these extracts [Cook *et al.* 1939]. Callow *et al.* [1939, 1, 2] have investigated independently a series of cases in which large doses of hormones were administered. One of these patients, a man under the care of Dr E. P. Sharpey-Schafer, was receiving 100 mg. of testosterone propionate daily. A clinical account of this case has been published elsewhere [Schrire & Sharpey-Schafer, 1939, case 3]. During the administration of testosterone propionate the excretion of 17-ketosteroids (measured colorimetrically by the modified Zimmermann reaction described by Callow *et al.* [1938]), and of androgenic activity, increased to about four times the level of that of a subsequent control period, when this excretion was within normal limits, so that there can be little doubt that the increase was directly caused by the injections; sufficient extract was available for chemical separation of the 17-ketosteroids, with which the urine was enriched, to be attempted.

The neutral fraction of the benzene extract of acid-hydrolysed urine was separated into ketonic and non-ketonic fractions by the Girard-Sandulesco reagent P. The ketonic fraction after chromatographic adsorption followed by elution of the column yielded androsterone and a compound which was identified as aetiocholan-3(α)-ol-17-one (Fieser's nomenclature [1937]; the "epi-oxy-aetiocholanon-17" of Ruzicka & Goldberg [1935]). A total of 60 mg. of crude androsterone and 58 mg. of crude aetiocholan-3(α)-ol-17-one were obtained from 7½ l. of urine or about 6½ days' output.

As a control, the extract from 50 l. of a bulk collection of normal men's urine was put through the same process. A total of 60 mg. of androsterone and 70 mg. of crude aetiocholan-3(α)-ol-17-one were separated. The isolation of the latter compound from normal urine has not previously been reported.

Thus from the patient receiving testosterone propionate 8 mg./l. of crude androsterone and 7·7 mg./l. of crude aetiocholan-3(α)-ol-17-one were isolated, while from the normal men's urine the same two compounds were obtained in yields of 1·2 and 1·4 mg./l. respectively. The androsterone which was isolated accounts for about 74% of the androgenic activity, assayed on capons, of the extracts of the "testosterone urine", and about 45% of the activity of the normal urine extract.

Experimental

All melting points in this paper were observed under the microscope on a slide on an electrically heated stage (Kofler's micro-melting point apparatus). The optical rotations were measured in absolute alcoholic solution in a 4 dm. tube.

Collection and extraction of urine. 24-hr. collections of urine were made for 8 days while the patient was receiving 100 mg. of testosterone propionate daily by intramuscular injection, beginning on the second day of injection. Toluene was used as preservative, and the completed collections were stored at 0° until hydrolysis, which was carried out not more than 5 days after collection. Before hydrolysis the *p*H of all the samples was 6, indicating that little, if any, putrefaction had taken place. The 24-hr. collections were hydrolysed, extracted, and separated into neutral, acidic, and phenolic fractions by the routine method in this laboratory [Callow, 1936]. Colorimetry indicated a content of 17-ketosteroids varying from 28 to 45 mg., average 38 mg. daily. By capon assay (for which I am indebted to Mr C. W. Emmens) the average androgen content was 126 I.U. per day.

The neutral fractions, equivalent to $6\frac{1}{2}$ days' excretion or $7\frac{1}{2}$ l., were combined and taken up in about 15 ml. of methanol. Next day the solution was filtered from traces of insoluble material; evaporation of the filtrate gave 0·54 g. of neutral fraction. Treatment with the Girard-Sandulesco reagent P (carbohydrazidomethylpyridinium chloride) [cf. Callow & Callow, 1938] gave a "ketonic fraction" weighing 0·255 g. and a "non-ketonic fraction" weighing 0·24 g. Colorimetry indicated that the ketonic fraction contained about 65 % of 17-ketosteroids.

Separation by fractional adsorption. The ketonic resin was dissolved in 10 ml. of carbon tetrachloride (A.R.), left overnight and filtered. The filtrate, diluted to about 25 ml., was put through a 26 × 1·5 cm. column of alumina (Merck's "Aluminium oxide standardized according to Brockmann") in carbon tetrachloride. It was developed in the usual way with about 1·5 l. of carbon tetrachloride, and then with carbon tetrachloride containing successively 0·1, 0·2 and 0·3 % of absolute alcohol. Successive portions of 200–500 ml. of the eluate were evaporated. The pure carbon tetrachloride eluate gave only a trace of coloured gummy material. The 0·1 % alcohol solution caused a narrow yellow band to move rapidly down the column, and evaporation of 300 ml. of eluate gave 11·7 mg. of gummy residue. Elution with the 0·2 and 0·3 % alcohol solutions gave crystalline residues. The weights, volumes of eluate, and the melting points and specific rotations of the residues are shown in Table I. After these fractions had been removed, no more solid was obtained by further elution with 0·3 % of alcohol in carbon tetrachloride, and subsequent treatment with 600 ml. of 0·5 % and 500 ml. of 1·0 % alcohol solutions yielded only 6 mg. of gummy material.

Table I

Fraction	Vol. and nature of solvent	Wt. crude crystals mg.	M.P. °C.	Optical rotation $[\alpha]_D$	Optical rotation $[\alpha]_{5461}$	Main product subsequently identified as
I	350 ml. 0·2 % EtOH in CCl_4	42	175–181	+92°	+104°	Androsterone
II	225 ml. ,, ,,	18	170–184 (recryst.)	+85°	+ 89°	,,
III	250 ml. 0·3 % EtOH in CCl_4	21·5	130–146	+89°	+124°	Aetiocholan-3(α)-ol-17-one
IV	225 ml. ,, ,,	15·6	115–151	+91°	+ 98°	,,
V	225 ml. ,, ,,	13	136–149	+102°	+122·5°	,,
VI	240 ml. ,, ,,	5·7	—	—	—	,,

Characterization of androsterone

The specific rotation, and the characteristic habit of subliming on to the coverslip during the determination of the M.P. on the micro-melting point apparatus suggested that both fractions I and II were androsterone. After two recrystallizations from methanol, fraction I yielded 24 mg. of crystals, M.P. 183–184° (soft at 179°). A mixed melting point with an authentic specimen of androsterone was 181–184·5°. The identity of the compound as androsterone was confirmed by preparation of the oxime. 10 mg. of the ketone, 6 mg. of hydroxylamine hydrochloride and 6 mg. of anhydrous sodium acetate were boiled under reflux in alcoholic solution for 2½ hr. The solvent was removed under reduced pressure, and the crystalline residue taken up in about 3 ml. of acetone, the solution filtered and evaporated to about 0·5 ml. On cooling 8 mg. of the oxime, M.P. 203–206 were obtained. After recrystallizing from acetone it had M.P. 206–212·5° and mixed M.P. with an authentic specimen of androsterone oxime 208–212·5°.

Fraction II after recrystallization from methanol had M.P. 182–184, which was not depressed by admixture with an authentic specimen of androsterone.

Identification of aetiocholan-3(α)-ol-17-one

The colour reaction of material from fraction III showed the absorptiometric spectrum characteristic of a 17-ketosteroid [cf. Callow *et al.* 1938]. 21 mg. of the crude fraction III were treated with a hot solution of 100 mg. of digitonin in 10 ml. of 50% aqueous alcohol. Next day the slight precipitate was removed by centrifuging and the supernatant liquid was diluted with water and extracted with ether; the ether extract, washed with water, and dried over sodium sulphate, yielded on evaporation a residue, 19 mg., which was recrystallized from aqueous methanol. The crystals showed a characteristic transition from a needle-shaped to a platy crystalline form at 137–139°, with partial fusion and resolidification. The final M.P. was 150°. Further recrystallization gave material with M.P. 147–151° and $[\alpha]_D + 100°$: $[\alpha]_{5461} + 130°$ (EtOH): and finally with M.P. 151–152° (transition point 140–142°). This M.P. was not depressed by admixture with an authentic specimen of aetiocholan-3(α)-ol-17-one, M.P. 152–153° (transition point 140°). Found: (Weiler) C, 78·2; H, 10·0%; calc. for $C_{19}H_{30}O_2$: C, 78·6; H, 10·4%.

Preparation of aetiocholan-3(α)-ol-17-one benzoate

Material, M.P. 147–151°, obtained by evaporation of the mother liquors from the last recrystallization of the above aetiocholan-3(α)-ol-17-one was dissolved in 0·25 ml. of dry pyridine and two drops of benzoyl chloride added. After heating on the water bath for 5 min., the mixture was cooled, and diluted with water drop by drop. The sticky crystals which separated were well washed with water, and recrystallized from aqueous methanol, from which characteristic bundles of needles, M.P. 140–160°, separated. After four recrystallizations from aqueous methanol, it had M.P. 153–161°. There was insufficient for further purification.

For comparison a specimen of aetiocholan-3(α)-ol-17-one benzoate, which has not been described before, was prepared from the authentic hydroxyketone as described above. After recrystallization from aqueous methanol, and from absolute methanol, it had a constant M.P. 161·5–163·5°, and crystallized well in long needles similar to those already described. The melting point of the sample prepared from the urine extract was not depressed by admixture with this authentic specimen.

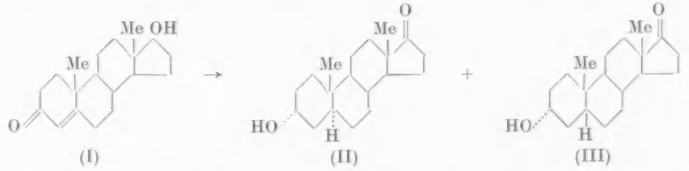
Fractions IV, V and VI were combined, after preliminary measurements of their melting points and specific rotations, and recrystallized from benzene. The top fraction partially melted and recrystallized at 137° and finally melted at 152°. The last fraction, obtained by evaporation of the mother liquors, M.P. 142–148°, yielded a benzoate, M.P. 151–160°. There was insufficient for further purification, but it was similar in crystalline form and behaviour to the substance identified above as aetiocholan-3(α)-ol-17-one benzoate. The top and intermediate fractions from the benzene recrystallizations were united (15 mg.) and treated with a hot solution of 80 mg. of digitonin in 10 ml. of hot 50% aqueous alcohol. After standing overnight there was only a slight precipitate, and 11 mg. of material were recovered from the filtrate. This was treated with acetic anhydride in presence of pyridine on the water bath for 30 min. The mixture was cooled, water was added and the oil which separated was extracted with ether; the extract was washed with aqueous Na_2CO_3 and with water, dried and evaporated. The residue, twice recrystallized from aqueous methanol, had M.P. 91–94·5°. The M.P. of a slightly impure authentic specimen of aetiocholan-3(α)-ol-17-one acetate (M.P. 89·5–94°) was not depressed by admixture with this specimen.

The normal urine extract used as a control was part of a bulk collection of 250 l. of normal men's urine worked up by Messrs Boots Pure Drug Co. This opportunity is taken to acknowledge their generosity in undertaking this work. The urine was collected with Brilliant Green as preservative, hydrolysed with HCl and extracted with chloroform. The chloroform was evaporated, and the extract sent to this laboratory, where it was taken up in benzene, and separated into neutral, acidic and phenolic fractions in the usual way. Colorimetry of the neutral fraction indicated 8 mg./l. of 17-ketosteroids, and capon assay 26 I.U./l. of androgenic activity. The neutral fraction of the extract from 50 l. of urine was separated into "ketonic" and "non-ketonic" fractions. These weighed 0·48 and 1·12 g. respectively. The ketonic fraction was taken up in carbon tetrachloride, and put through a column of alumina. The column was developed with carbon tetrachloride, and carbon tetrachloride containing 0·1, 0·2 and 0·3% of absolute alcohol, as described for the "testosterone urine". Development with any one solvent was continued until evaporation of about 250 ml. of eluate gave a residue weighing less than 10 mg. Evaporation of 310 ml. of the 0·1% alcohol eluate gave 49 mg. of a semicrystalline residue. After treatment with charcoal and repeated recrystallization from aqueous methanol, 6 mg. of a product, M.P. 142–150°, were obtained. This was subsequently identified as *trans*dehydroandrosterone. 15 mg. of material from the mother liquors were treated with 60 mg. of digitonin in 70% aqueous alcohol. The precipitated digitonide was decomposed in the usual way with pyridine, and yielded 2·5 mg. of a crystalline substance, M.P. 136–145°. This was combined with the material obtained by fractional crystallization and converted into the benzoate. This, after recrystallization from ethyl acetate, was recognized as *trans*dehydroandrosterone benzoate. It then had M.P. 242–248°, not depressed by admixture with an authentic specimen. Elution with a total of 810 ml. of 0·2% alcohol in carbon tetrachloride yielded 60 mg. in all of crystalline residue. This was identified as androsterone by its M.P., mixed M.P. and property of subliming. The next fraction, obtained by elution with 370 ml. of 0·3% alcohol in carbon tetrachloride, yielded 70 mg. of crystalline material. After repeated recrystallization from aqueous methanol, the M.P. became constant at 144–147°. A mixed M.P. with an authentic specimen of aetiocholan-3(α)-ol-17-one was 145–150°. The identity of the compound was confirmed by preparation of the benzoate. This had M.P. 159–162°, and a mixed M.P. 162–163·5° with an authentic specimen of aetiocholan-3(α)-ol-17-one benzoate.

Discussion

The isolation of androsterone and the stereoisomeric compound aetiocholan-3(α)-ol-17-one from the urine of a man receiving testosterone propionate, and, in much smaller quantities, from normal men's urine, is particularly interesting from the point of view of determining the parent substances of the steroid compounds excreted in urine, whether the former have their origin in the gonads or the adrenals. Testosterone is the only androgen which has been isolated from gonadal tissue, but there is no evidence that it is ever excreted in urine. This work owed its inception to the hope that some degradation product might be recognized as an index of testosterone production, just as pregnanediol is recognized as an index of progesterone production [cf. Venning & Browne, 1936; 1937; Venning, 1937]. Since androsterone has been isolated from normal women's urine [Callow & Callow, 1938] and aetiocholan-3(α)-ol-17-one from the urine of a woman with adrenal hyperplasia [Butler & Marrian, 1938], the meaning of the excretion of these two compounds must remain a matter of speculation until further work has been done. The possible occurrence of aetiocholan-3(α)-ol-17-one in the urine of normal women is now being investigated.

Hartmann & Locher [1935] suggested that pregnanediol and *allo*pregnanediol, which can be isolated from the urine of pregnant women, were derived from progesterone by reduction. A completely analogous process in ring I can be postulated for the degradation of testosterone (I) to androsterone (II) and aetiocholan-3(α)-ol-17-one (III), involving the reduction of the 3-keto-group to give a 3(α)-hydroxy compound, and of the 4:5 double bond to give both the two possible configurations at position 5, with oxidation of the 17-hydroxyl group.



Butler & Marrian [1938] suggested that the aetiocholan-3(α)-ol-17-one which they isolated from the urine of a woman with adrenal hyperplasia was derived by partial oxidation from pregnane-3(α):17:20-triol, which they isolated from the same urine. The finding of the former compound as a degradation product of testosterone does not, of course, disprove this hypothesis, but it shows that another mechanism is possible.

The degradations of testosterone to androsterone and aetiocholan-3(α)-ol-17-one now experimentally demonstrated may be compared with the speculative scheme of degradation of male hormones in the body recently put forward by Marker [1938]. He suggested that androsterone and aetiocholan-3(α)-ol-17-one were stages in the reduction of Δ^4-androstene-3:17-dione. This scheme would be in accordance with the facts, provided that Δ^4-androstene-3:17-dione were derived from testosterone, and not the reverse, as assumed by Marker. He also suggested that *trans*dehydroandrosterone (Δ^5-androsten-3(β)-ol-17-one) is derived from Δ^4-androstene-3:17-dione. In the case investigated there was actually no large increase in the excretion of *trans*dehydroandrosterone during the administration of testosterone propionate. From the behaviour of the extract from normal urine, it is known that this compound is eluted from the adsorption column with carbon tetrachloride containing 0·1 % of alcohol. The

corresponding fraction from the "testosterone urine" was a gum which weighed only 11·7 mg., and was obviously a mixture. The yield of this fraction from seven times the volume of normal men's urine was 49 mg. of semicrystalline material, from which 8 mg. of *trans*dehydroandrosterone were isolated. *trans*Dehydroandrosterone is more probably derived from the adrenal cortical secretions [cf. Callow, 1938].

When this work was nearing completion it was learnt from Prof. J. W. Cook that he had isolated androsterone as a degradation product of administered testosterone, and accounts of both his and our investigations were given to the meeting of the Biochemical Society on 10 February 1939 [Cook *et al.* 1939; Callow *et al.* 1939, 1].

SUMMARY

Androsterone and aetiocholan-3(α)-ol-17-one have been isolated from the urine of a man receiving 100 mg. of testosterone propionate daily in yields of 8 and 7·7 mg./l., respectively. The same two compounds have been obtained from normal men's urine in yields of 1·2 and 1·4 mg./l.

I have pleasure in thanking Dr E. P. Sharpey-Schafer of the British Post-Graduate Medical School for putting the urine from his case at my disposal, and Dr D. Beall for arranging its collection and transport. I am greatly indebted to Prof. Ruzicka for a specimen of aetiocholan-3(α)-ol-17-one, which he worked up from the semicarbazone so that a comparison with the natural product might be made, and to Prof. G. F. Marrian for a specimen of the acetate. Finally I have to acknowledge much encouragement and helpful criticism from my husband, Dr R. K. Callow.

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LXX. THE PHOSPHORYLATION OF CARBOHYDRATE IN LIVING CELLS

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It has been shown previously that the fermentation of sugar by living yeast is accompanied by a decrease in the amount of orthophosphate present in the cell as compared with the resting value, and that this decrease persists until the sugar added has been fermented, at which stage the orthophosphate tends to resume its original value [Macfarlane, 1936]. This coincidence of phosphorylation with fermentation provided evidence of what had hitherto been an assumption, namely, that the fermentation of sugar by living yeast takes place in a manner analogous to that found in yeast juice and other cell-free fermentations, through the intermediate formation and decomposition of hexosephosphoric esters. The scale and technique of the previous experiments were not suitable for the identification of any phosphoric esters formed; by working with larger quantities it has now been established that hexosediphosphate is formed during the normal fermentation of sugar by living yeast and that phosphoglyceric acid is also present when the normal fermentation has been inhibited by the addition of NaF. The experiments to be described also illustrate several points of difference between the fermentation of the living cell and that of the dead or cell-free preparation which are interesting in the general question of glycolysis by living cells.

Phosphoric esters formed in living yeast during fermentation

Mild ale yeast was obtained fresh from the brewery vats, washed three times with water and well drained on Büchner funnels. The yeast was used without further pressing, and the water content therefore varied somewhat in different batches. The fermentations were carried out at room temperature in large bottles attached to 500 ml. nitrometers filled with brine. Weighed amounts (100–400 g.) of yeast were mixed with twice their weight of water and the contents of the bottle saturated with CO_2 before connexion to the nitrometers. After an interval of 10 min. to allow equilibrium to be established, the following additions were made to different bottles: (*a*) water only; (*b*) NaF, in final concentration 0·05 *M*; (*c*) sugar, 10 g. per 100 ml. water; (*d*) sugar, followed by NaF in final concentration 0·05 *M* after the maximum fermentation rate had been established. Enzyme action was stopped by addition of 50 ml. 20% trichloroacetic acid per 100 g. yeast. The precipitate was separated after ½ hr. by filtration and washed with 5% trichloroacetic acid. From the combined filtrate and washings a sample was removed for P estimations by Briggs's colorimetric method. The total acid-soluble P was determined after ashing, and the content of "pyro" and organic P was calculated from the hydrolysis curve in *N* HCl at 100° according to the equations:

$$\text{"Pyro" P} = \Delta\,(7\text{ min.} - 0\text{ min.}) - \Delta\,(30\text{ min.} - 7\text{ min.}),$$

$$\text{Organic P} = \text{total P} - (\text{ortho P} + \text{"pyro" P}),$$

$$\text{Hexosediphosphate P} = \Delta\,(180\text{ min.} - 7\text{ min.}) \times 1{\cdot}5.$$

Alkali-labile P was estimated as the increase of ortho-P after 15 min. in *N* NaOH at 22°.

The bulk of the filtrate was treated with an amount of Ba acetate equivalent to the total P present, followed by $Ba(OH)_2$ to *p*H 8·0 and one-tenth volume of alcohol. The precipitate was removed by filtration; the filtrate was treated with basic Pb acetate, and the Pb precipitate removed, decomposed with H_2SO_4 and reconverted into the Ba salt. From P estimations in the successive filtrates the amount of P present in (*a*) sparingly soluble Ba salts, (*b*) soluble Ba salts, (*c*) soluble Ba salts with insoluble Pb salts was calculated by difference.

Identification of hexosediphosphate. The sparingly soluble Ba salt consists to a large extent of inorganic (ortho-, meta- and pyro-) P, together with any hexosediphosphate, phosphoglycerate etc. The crude salt was purified by repeated solution in 10 parts H_2O acidified with HCl to *p*H 3·6 and reprecipitation, at first as the acid Ba salt by addition of 3 vol. alcohol (the inorganic P tending to remain in solution) and finally by neutralization of the aqueous solution with $Ba(OH)_2$. The purification was such as to remove any trace of hexosemonophosphate but owing to the overlapping solubilities and the large excess of pyrophosphate it was exceedingly difficult to obtain a sample of hexosediphosphate free from inorganic P. Proof of the presence of this ester was therefore obtained by the preparation from these fractions of the crystalline phenylhydrazine salt of 6-phosphoglucosazone, which is formed from hexosediphosphate with elimination of one P atom.

Protocol, Yeast 31. Fermentation of fructose by 650 g. yeast. The sparingly soluble Ba precipitate, wt. 7·8 g., contained 786 mg. total P, of which 133 mg. were organic. The acid Ba salt was reprecipitated five times with alcohol and finally precipitated by neutralization of the aqueous solution. Wt. 0·81 g. Total P 9·3 %, ortho-P nil. Reducing power (Hagedorn and Jensen) 7·6 %; ketose (Seliwanoff) 5 %. $[\alpha]_{5461}+1·8°$. Ba hexosediphosphate has a reducing power (Hagedorn and Jensen) of 12 % and a Seliwanoff value of 10 %. Calculated from the hydrolysis curve in *N* HCl at 100°, 72 % of the total P content was hexosediphosphate-P.

Formation of 6-phosphoglucosazone. 0·2 g. pure Ba hexosediphosphate was converted into the free acid, vol. 5 ml., heated with 0·5 ml. phenylhydrazine and 1·0 ml. glacial acetic acid at 100° for 15 min. and cooled in ice for 20 min. The yield of 6-phosphoglucosazone (phenylhydrazine salt) was 70 % of the theory, with 43 % recovery on recrystallization from hot alcohol and chloroform; M.P. 153–4°. 0·33 g. of the Ba salt described above (31 mg. P), treated similarly, gave 151 mg. osazone, which was recrystallized with 45 % recovery; M.P. 153·6°; P=5·71 %; required for $C_{24}H_{31}O_7N_6P$, P 5·68 %. Calculated on the basis of a 70 % yield of phosphosazone, 74 % of the total P in the salt was present as hexosediphosphate, which is in good agreement with the other analytical figures.

Estimation of hexosediphosphate. The purification of the hexosediphosphate fraction outlined above entails a considerable loss of organic P and is therefore not suitable for quantitative work, while calculation from the hydrolysis curve of a crude salt containing much labile P involves a considerable error. Attempts to prepare the osazone directly from the crude salt were unsatisfactory. For the routine estimation of hexosediphosphate, therefore, the crude Ba salt was purified once as the acid salt and precipitated as the neutral salt from aqueous solution. The crystalline phospho-osazone was then prepared from an aliquot sample of the salt under standard conditions, and the amount of P as hexosediphosphate was calculated from the weight of osazone obtained on the basis of a 70 % yield.

Table I. *Distribution of acid-soluble phosphorus in yeast*

	Autofermentation				Sugar fermentation					Sugar fermentation inhibited by NaF				
Exp. no.	29	32	39	39	29	30	31	32	37	29	39	30	32	29
Additions*	—	—	—	NaF	F	F	F	G	M	F	F	F	G	F
Total duration of exp. (min.)	0	20	20	30	25	20	25	20	35	30	30	40	50	65
Duration of exp. after NaF	—	—	—	10	—	—	—	—	—	10	10	20	30	45
CO_2 evolution, ml./5 min./100 g. yeast	—	—	15	0	180	150	135	170	150	<12	< 5	<12	0	0
mg. P per 100 g. yeast:														
Total acid-soluble P	178	230	205	202	175	164	190	224	153	178	191	174	220	190
Ortho-P	76	81	80	70	35	38	45	43	25	22	37	21	23	7
"Pyro"-P	63	111	82	73	64	—	92	115	68	58	75	—	102	77
Organic P	39	39	43	60	76	73	53	67	60	98	78	98	94	105
(*a*) Insoluble Ba ppt.	13	13	15	30	37	35	22	28	23	46	31	48	54	35
Hexosediphosphate P†	—	1	—	—	—	—	9·6	7·8	11	—	—	—	3	—
Phosphoglyceric P‡	0	—	0	0	0	0	—	0	—	6	5	7	—	7
(*b*) Soluble Ba salts	26	25	28	30	39	38	31	29	36	52	47	50	40	57
P pptd. by basic Pb acetate	—	6	—	—	—	12	3	11	—	—	—	17	22	—
(*c*) Alkali-labile P	—	0·7	—	—	—	—	—	0·9	—	—	—	—	1·4	—

* F = fructose; G = glucose; M = mannose.

† Calc. from weight of crystalline osazone obtained from the purified salt on basis of a 70% yield.

‡ Calc. from weight of crystalline BaH phosphoglycerate.

Identification of phosphoglyceric acid. Phosphoglyceric acid was isolated quantitatively from the crude sparingly soluble Ba salts as the crystalline acid Ba salt. It was found advantageous to heat the salt first in *N* HCl at 100° for ½ hr. to decompose labile P compounds. The *p*H of the solution was then brought to 3·0 with $Ba(OH)_2$ and 2 vol. alcohol added. The precipitate was dissolved in dilute HCl and 2 vol. alcohol were cautiously added; if any phosphoglyceric acid was present the characteristic crystalline BaH salt was rapidly deposited.

Other phosphoric esters. It was anticipated that the fraction with soluble Ba salts would contain some hexosemonophosphate, together with phosphopyruvate etc. Only a small part of the P present, however, was precipitated with basic Pb acetate and this fraction, after reconversion into the Ba salt, contained only 1–3 % P. In spite of repeated attempts at fractionation by Hg acetate, crystallization of brucine salts or alcohol fractionation, the constituents of this mixture could not be separated sufficiently for the identification of any phosphoric ester and no crystalline phenylhydrazine derivative could be obtained.

The values obtained for the distribution of the acid-soluble phosphorus during different types of fermentation are shown in Table I.

Effect of sugar fermentation on P distribution. During autofermentation the greater part of the organic P is in the fraction with very soluble Ba and Pb salts, probably of nucleotide nature, but a trace of hexosediphosphate is present. During the fermentation of sugar, whether fructose, glucose or mannose, the organic P is increased in amount, the increase being distributed almost equally between the insoluble and soluble Ba salts. In the insoluble fraction only 50 % of the increase was accounted for by the estimated increase in hexosediphosphate-P (10 mg. P per 100 g. yeast); the method of estimation of this ester however almost certainly gives too low values, since no allowance was made for loss in purification or by the hexosediphosphate carried into the soluble fractions. No phosphoglyceric acid could be isolated either from the resting or fermenting yeast and the amount of alkali-labile P was only slightly raised during fermentation.

Effect of NaF on distribution of P. When the fermentation of sugar by living yeast was inhibited by the addition of NaF the presence of phosphoglyceric acid was readily established; the amount found in different batches of the same yeast was constant, *c.* 7 mg. P per 100 g. yeast, and was not appreciably raised by prolonging the experiment with the NaF-poisoned yeast, e.g. from 10 to 45 min. The ortho-P content however slowly decreased in such yeast (Table I, Yeast 39); this appears to be due to the continued formation of phosphoric esters from glycogen present in the cell, since it was also observed in autofermentation in presence of NaF.

Inhibition of glycolysis with varying concentrations of fluoride

In the foregoing experiments 0·05 *M* NaF was used, since it was found that with large quantities of yeast this concentration was necessary to produce complete inhibition: even so the inhibition was rarely complete in less than 10 min. The effect of various concentrations of NaF on the fermentation rate is shown in Table II. *M*/200 fluoride caused only 41 % inhibition of glycolysis in the cell, though in yeast juice or muscle extract more than 90 % inhibition is produced by this, or even smaller, concentrations. The sensitivity of glycolysis in different tissues to NaF was shown by Dickens & Šimer [1929] to be extremely variable; in view of the disparity between yeast cells and cell-free extracts the sensitivity may be interpreted as a characteristic of the tissue rather than of the glycolytic enzyme.

Table II. *Percentage inhibition with varying concentrations of fluoride*

2 g. fresh yeast + 9 ml. 10% glucose were fermented 15 min. at 25°; 1 ml. NaF was added and the average fermentation rate measured during the next hour.

NaF molarity	ml. CO_2/5 min.	% inhibition
0	6·3	—
0·004	4·2	33
0·005	3·7	41
0·006	3·0	52
0·008	1·6	74
0·01	0·6	91
0·02	0	100

Time relationship between esterification and fermentation in living yeast

The coincidence of phosphorylation and fermentation has been studied more closely, particularly in the initial and final stages of fermentation of a limited quantity of sugar.

A suspension was made by mixing one part washed fresh yeast with 5 parts water, some of which (A) was used immediately and some (B) after 24 hr. at room temperature. 10 ml. samples of the suspension were placed in fermentation flasks, saturated with CO_2 and connected to nitrometers. After 10 min. incubation, 1 ml. 10% glucose was added and the fermentation rate measured over short intervals. The fermentation was stopped in individual flasks at short intervals by the addition of 3 ml. 20% trichloroacetic acid, and the orthophosphate content of the filtrate estimated. The complete fermentation of 100 mg. glucose would cause the evolution of 25 ml. CO_2; in the experiment quoted in Table III the rate of fermentation fell, from the maximum of 1·1 ml. CO_2/2 min.

Table III. *Time relationship between esterification of inorganic P and CO_2 evolution in living yeast*

Yeasts: A, fresh; B, after 24 hr.

Time after addition of glucose	CO_2 rate ml./2 min.		Ortho-P mg./2 g. yeast		Time after addition of glucose min.	CO_2 rate ml./2 min.		Ortho-P mg./2 g. yeast	
	A	B	A	B		A	B	A	B
0	—	—	1·35	1·66	40	0·7	0·9	—	0·92
15 sec.	0	—	1·34	—	46	0·7	0·8	—	0·86
30 ,,	0	—	1·30	—	48	0·6	0·7	1·01	0·84
1 min.	0	0	1·27	—	50	0·2	0·6	—	0·92
2 ,,	0·1	0	1·10	—	54	0·2	0·4	—	1·18
3 ,,	0·2	0	0·93	—	58	0·1	0·2	—	1·12
5 ,,	0·5	0·3	0·80	—	60	<0·1	0·1	1·09	—
10 ,,	1·1	0·7	0·75	—	62	—	<0·1	—	1·12
20 ,,	1·1	1·0	0·83	—	70	—	—	—	1·17
30 ,,	0·9	0·9	—	0·88					

to the autofermentation rate of less than 0·1 ml. CO_2/2 min. when approximately 80% of the sugar had been fermented. It will be seen that 3 min. after the addition of glucose 0·42 mg. inorganic P (equivalent to 2·4 mg. glucose as hexosemonophosphate) had disappeared, but that the fermentation did not reach its maximum rate until after the esterification was maximal. The marked lag in the attainment of the maximum rate of CO_2 evolution (Table III) is reminiscent of the Gäransteig period in fermentation by maceration extracts [Meyerhof, 1918] during which hexosediphosphate is formed. The decrease in orthophosphate in living yeast was maximal in less than 5 min., the level then

remaining steady for ½ hr., till, with the consumption of the sugar, the CO_2 rate slackened and finally ceased and the inorganic P rose towards its original value.

The initial value of orthophosphate in yeast B (Table III), i.e. after 24 hr. "resting", was decidedly higher than in the fresh sample; the final value after fermentation was the same in both samples and less than the original value in the fresh yeast. It was found that if a second addition of glucose were made to the yeast, the time taken to attain the maximum rate increased with the interval between the two fermentations; during this interval there was a slow rise in orthophosphate. The point has not been investigated in great detail, but the experiments indicated a "rejuvenating" effect of the fermentation in building up labile P compounds which are slowly hydrolysed in the "resting" cell.

Independence of orthophosphate content of the cell and that of the medium

In the experiments so far cited the fermentations were carried out with mixtures of yeast, water and sugar, so that the observed changes in P distribution were internal. The following experiment demonstrates that this change is independent of the concentration of P in the surrounding medium. Weighed amounts (0·5 g.) of yeast were placed in centrifuge pots to which either H_2O, or phosphate buffer *p*H 6·6, or 1 ml. 10 % glucose or glucose + phosphate was added, the volume in each case being made up to 3 ml. with water. The yeast was well mixed and kept at 20° for 20 min. After centrifuging, the supernatant was separated as quickly and completely as possible with a capillary pipette, and the residue extracted with 5 ml. 0·5 % trichloroacetic acid. Inorganic P was then estimated in the supernatant liquid (external P) and in the trichloroacetic acid extract (internal P). It will be seen (Table IV) that in the presence of glucose,

Table IV. *Internal and external P concentrations in fermenting yeast*

0·5 g. yeast in 3 ml. solution.

Additions	mg. ortho-P	
	Supernatant liquid	Trichloroacetic acid extract
(1) H_2O	0	0·46, 0·46
(2) Glucose	0	0·32, 0·32
(3) 0·36 mg. P as phosphate buffer	0·358	0·48
(4) 0·36 mg. P as phosphate buffer + glucose	0·358	0·36
(5) 0·18 mg. P as phosphate buffer	0·178	0·46
(6) 0·18 mg. P as phosphate buffer + glucose	0·178	0·33

i.e. during fermentation, a decrease in orthophosphate takes place inside the yeast cell, while the concentration in the surrounding fluid remains unchanged. It has been shown by Hevesy *et al.* [1937], by means of experiments with radioactive P compounds, that there is no transference of P between the yeast cell and the medium unless growth is taking place.

The cycle of phosphate in glycogen and glucose fermentations in the cell

The experiments which have been described illustrate the general principle that in the decomposition of a substrate to its normal end products by a living cell there is no unlimited accumulation of intermediate products but a constant and perhaps extremely low "working level" of such substances. For instance in fermentation by living yeast the concentration of phosphoric esters is constant and independent of the total amount of CO_2 produced; this is in great contrast to fermentation in yeast juice, where in presence of excess glucose and phosphate the

amount of esterified P found is in stoichiometric proportion to the CO_2 evolved [Harden & Young, 1906].

The evidence suggests that there are two cycles with many points in common in which P is concerned in the yeast cell; firstly in the slow fermentation of glycogen present in the cell (autofermentation) and secondly in the rapid fermentation of hexoses presented to the cell. The mechanism of glycogen esterification in cell-free extracts is known to differ from that of glucose; esterification takes place in the absence of cozymase [Nilsson, 1930] and is not inhibited by fluoride [Meyerhof, 1926] or by iodoacetate [Macfarlane, 1931]. Since it is not possible to remove the preformed glycogen to ascertain the true resting level of the P the only evidence that glycogen is being esterified in the living cell is the fact that in presence of NaF esterification of inorganic P takes place. No phosphoglyceric acid was isolated from the autofermentation in presence of NaF, though it was readily obtained from an equal quantity of yeast after fermentation of sugar for the same length of time (Table I, Exp. 39).

The course of events during the fermentation of hexoses in the yeast cell is consistent with the following picture, based on the relationships between P-transference and oxidoreduction reactions which have been demonstrated in cell-free extracts [see Meyerhof *et al.* 1937; Needham & Pillai, 1937]. In the resting cell there is a small amount of hexosediphosphate (Table I) and of adenylic acid, some of which is present as adenylpyrophosphate [Wagner-Jauregg, 1936]. Part of the added hexose is therefore rapidly esterified by transference of P from adenylpyrophosphate and gives rise to triosephosphate by the reactions

$$\text{hexosemonophosphate} \rightarrow \text{hexosediphosphate} \rightleftarrows \text{triosephosphate}.$$

The coupled reaction

$$\begin{aligned} &\text{triosephosphate} + \text{acetaldehyde} \rightarrow \text{phosphoglyceric acid} + \text{alcohol} \\ &\text{glucose} + H_3PO_4 \qquad\qquad \rightarrow \text{hexosemonophosphate} \end{aligned}$$

begins to take place, the rate of the oxidoreduction and consequently of the esterification of inorganic P rising as the concentration of triosephosphate increases autocatalytically, until the limiting concentration of phosphoric esters is reached and the fermentation rate becomes maximal (see Table III). Once this rate is established the rate of breakdown of the phosphoric esters is in fact equal to the rate of their formation, since the level of P remains constant; when the free sugar has disappeared however it is probable that the reaction

$$\text{phosphopyruvic acid} + \text{adenylic acid} \rightarrow \text{pyruvic acid} + \text{adenylpyrophosphate}$$

no longer predominates in the decomposition of phosphopyruvic acid but that instead the reaction

$$\text{phosphopyruvic acid} \rightarrow \text{pyruvic acid} + H_3PO_4$$

leads to the reappearance of inorganic P in the cell.

The addition of NaF to the fermenting yeast inhibits the breakdown of phosphoglyceric acid to phosphopyruvic acid and consequently both the formation of acetaldehyde and the reformation of adenylpyrophosphate are suppressed. The ultimate effect of fluoride therefore is that the fermentation cycle is immobilized so soon as all the cozymase present has been reduced by triosephosphate, with formation of phosphoglyceric acid. Since the latter reaction is stoichiometric, the amount of phosphoglyceric acid formed in the fluoride-poisoned yeast cell should be dependent on the cozymase present, but independent of the duration of the reaction. The latter was found to be the case (Table I) while the amount of "pyro"-P found in corresponding batches of

yeast was appreciably less in the presence of fluoride than in the normal fermentation, as would be expected if reformation of adenylpyrophosphate had been inhibited. Approximately 7 mg. P per 100 g. yeast were isolated as phosphoglyceric acid, equivalent to 140 mg. cozymase. Meyerhof & Ohlmeyer [1937] estimated the cozymase content of bottom yeast as 50 mg. per 100 g., a figure based on the amount in boiled extracts, which may not represent a complete extraction. The value calculated above is only in fair agreement, but no figures are available for the actual cozymase content of the yeast used. It seems probable that in a normal fermentation this figure (7 mg. P/100 g. yeast) represents the maximum sum of the P present as 3- and 2-phosphoglyceric and phosphopyruvic acids, since for the formation of further quantities of phosphoglyceric acid the decomposition of an equivalent amount of phosphopyruvic acid is necessary.

Out of the seven phosphoric esters which are known to be intermediate products in the fermentation of sugar by yeast juice only hexosediphosphate was identified with certainty in the normal fermentation of the cell. Since the increase in organic P was of the order of 30 mg. P per 100 g. yeast of which at least 10 mg. were present as hexosediphosphate, there was an accumulation of this ester relative to the other esters. It may be surmised that this ester acts as a pressure head, maintaining by an enzymic equilibrium reaction [Meyerhof & Lohmann, 1934] the optimum supply of triosephosphate.

The detection of phosphorylation in tissues

It is clear that if the initial concentration of adenylpyrophosphate present in the tissue is so high that sufficient phosphoric ester to saturate the enzymes can be produced by direct phosphorylation of the added sugar then the esterification or even the presence of inorganic P is unnecessary. When therefore the principle is recognized that glycolysis in the living cell takes place by a cycle of P transference in which the actual amount of P involved approaches catalytic dimensions, the question of practical importance for the detection of such phosphorylation is not how much but how little P need be esterified at any one time in order to provide the concentration of phosphoric esters necessary for the maximum rate.

It is known that in certain isolated tissues [Boyland & Boyland, 1935] adenylpyrophosphate is very rapidly broken down by the enzymes present. It appears probable that if a tissue is fresh and only recently removed from its natural source of carbohydrate in the body, or if the experimental procedure necessary involves little injury, the amount of inorganic P esterified is likely to be extremely small.

This point is illustrated by observations on the orthophosphate concentration during glycolysis by a young culture of *Bact. coli*. The culture was obtained by successive tenfold dilutions with 1% trypsin-broth of 0·5 ml. of a fresh subculture after 15, 7 and $6\frac{1}{2}$ hr. incubation; the bacteria were separated from the broth by centrifuging $9\frac{1}{2}$ hr. after the last transfer, washed twice with 0·086% NaCl and suspended in saline. The orthophosphate content during incubation in absence and presence of glucose was then estimated. It will be seen (Table V) that the initial value of the orthophosphate and the actual decrease in presence of glucose were both very small, and that here also as in living yeast the P values increased as the glucose was consumed. The lowest amount of P actually estimated in these experiments was 0·004 mg., with an accuracy of approximately $\pm 0{\cdot}0005$ mg. P. In the absence of glucose there was a rapid increase in inorganic P, presumably by autolytic changes in an unfavourable medium; by comparison with these values the decrease during glycolysis was marked, and quite outside the experimental error.

Table V. *Inorganic P during glycolysis by* Bact. coli

2 ml. bacterial suspension (15·6 mg. dry wt. in 0·05 *M* $NaHCO_3$, 0·015 *M* NaCl) + 1 ml. glucose or water incubated at 37°; + 3 ml. 5% trichloroacetic acid. 4 ml. samples for P estimations.

Time min.	No sugar mg. P	Sugar added	
		mg. P	mg. glucose
0	0·009	0·009	9·9
30	0·023	0·006	6·5
60	0·027	0·006	3·9
120	0·033	0·014	0·7

In experiments on the glycolysis of retina Bumm & Fehrenbach [1931] found "in glukose-freier Ringer-Lösung immer etwas mehr Phosphat abgespalten als im Ansatz mit Glukose", though the glycolysis could take place even if no detectable inorganic P were present in the tissue. The differences observed were of the order 0·002–0·004 mg. P per mg. dry wt. of retina. The glycolytic rates of yeast, *Bact. coli* and retina are high, while the observed differences of P concentration between fermenting and non-fermenting cells are of the order 1–2 mg. P/g. dry wt. If the amount of P esterified is a function of the glycolytic rate the detection of phosphorylation in tissues of low glycolytic rate will require an extremely delicate technique; especially if the tissue examined is not readily available in large amounts.

Generally speaking the rapid formation of inorganic P in an isolated tissue indicates a physiological lesion. The retina, which is often classed as having an "abnormally high" glycolytic rate, is one of the few tissues which can be detached with little injury and used entire in a manometric experiment; it is interesting to note that in the experiments of Bumm & Fehrenbach the glycolytic rate (mg. lactic acid/mg. dry wt./hr.) averaged 0·234 in 9 exps. where no inorganic P was detectable and only 0·14 in 8 exps. in which the inorganic P had increased to 0·02 mg./mg.

The formation of glycogen in living yeast. The occurrence of an induction period in the fermentation of sugar by living yeast has been pointed out by Willstätter & Rohdewald [1937] who found a marked increase in glycogen during this period and therefore advanced the view that glucose is not directly fermented but is first transformed into glycogen. The rapid formation of glycogen by fresh yeast has been confirmed by Goda [1938] who however does not support Willstätter & Rohdewald's hypothesis, since he found that in old yeast there was a rapid fermentation of glucose but no parallel formation of glycogen. It seems clear that glucose is directly fermented by yeast juice since the sugar is fermented more rapidly than glycogen either present in or added to the juice; it is difficult to imagine how the juice obtains this property if it is not also one of the living cell, or why glycogen newly formed from added glucose should be more rapidly fermented than glycogen preformed in the cell. Though no glycogen estimations were made in the present experiments, the experimental conditions were very similar to those of the previous workers; it seems probable therefore that the esterification of inorganic P and the formation of glycogen are contemporaneous. It was suggested by Robison [1932] that esterification of sugar might take place by a preliminary enolization and phosphorylation on carbon atom 1 of the enol sugar, followed by migration of the P group to carbon atom 6. The discovery however that the fermentation of mannose by yeast preparations may result in the almost exclusive accumulation of mannosephosphate [Jephcott & Robison, 1934] and that muscle and yeast extracts contain an enzyme, phosphohexokinase [Lohmann, 1931; Tankó & Robison, 1935] which can transform the individual

hexose-6-phosphates into an equilibrium mixture of aldose and ketose esters, indicates that enolization takes place in the phosphorylated rather than in the free sugar. Glucose-1-phosphate, which is an intermediate product in glycogen breakdown, can also be transformed enzymically into the equilibrium mixture of 6-phosphoesters [Cori *et al.* 1937]. It seems possible that the fermentable sugars are primarily phosphorylated on the reducing group, and that the observed formation of glycogen in living yeast takes place by condensation of glucose-1-phosphate with elimination of phosphate. It would be of interest to know the behaviour of fructose under similar circumstances.

Non-phosphorylating glycolysis

The demonstrated changes in P distribution during fermentation in the living yeast cell are so consistent with the theory of alcoholic fermentation developed from experiments on cell-free extracts [see Meyerhof *et al.* 1937] that there can be little doubt that the processes are analogous. Yet there are a number of differences between the cell and the cell-free extract which may be summarized as follows: fresh yeast does not ferment added glycogen or hexosediphosphate; the rate of fermentation of sugar is not increased by inorganic P, cozymase or arsenate; there is no stoichiometric relationship between the CO_2 evolved and the actual decrease in inorganic P. These apparent inconsistencies may be ascribed to the fact that the cell is a well-concerted enzyme system protected by a selectively permeable membrane, so that the possession of these negative attributes by other tissues cannot furnish any evidence either for or against the existence of a phosphorylating glycolysis in these tissues. It is, however, these attributes which have been advanced as the primary experimental basis of the hypothesis of non-phosphorylating glycolysis [see Ashford & Holmes, 1929; Ashford, 1934; Needham & Nowinski, 1937; Needham *et al.* 1937]. The conclusions which have been drawn in such cases are frequently due to the expectation, either implicit or avowed, that an intact tissue or tissue slice will behave like a cell-free extract of muscle or yeast, whereas they may be expected to differ from such extracts in the same way as living yeast. For instance, the fact that the glycolysis of retina is not accelerated by phosphate or arsenate [Bumm & Fehrenbach, 1931] is more reasonably interpreted as a similarity between this tissue and living yeast than as a dissimilarity from glycolysis in yeast juice.

The demonstration of esterification during glycolysis is experimentally more difficult in tissues such as brain, kidney, tumour and chick embryo which show a rapid autolytic formation of inorganic P than in yeast where the formation of inorganic P in the resting cell is negligible in short experimental periods. In the former cases a small and limited decrease in orthophosphate due to esterification in glycolysis may be opposed to an increase due to hydrolysis by phosphatases of cell substrates which may perhaps have no connexion with the formation of lactic acid such as aminoethylphosphate or diphosphoglycerate.

Nevertheless the esterification may still be detectable; thus in the experiments of Ashford [1934] with brain tissue, the inorganic P per 100 g. tissue was 10–25 mg. less after incubation in the presence of glucose than in its absence, though both values were much greater (30–40 mg. P) than the zero value. This is indirect evidence of phosphorylation during glycolysis; the fact that the esterification was independent of the total glycolysis is consistent with the theory of a phosphorylating cycle.

The experiments of Needham *et al.* [1937] with chick embryo do not provide any evidence on this point, since no values are given for the orthophosphate in embryos incubated without glucose, a necessary control where a rapid hydrolysis

of phosphoric esters is shown to be taking place. Their values for the P distribution during glycolysis in absence and presence of fluoride show a relative increase of esterification in the latter case, particularly in the phosphoglyceric acid and hexosediphosphate fractions.

Thus in one experiment (Table XIV, *loc. cit.*) the distribution of P (in % of the acid-soluble P) was: inorganic P 48·5 % and 34·7 %, insoluble "resistant" ester P 0·7 % and 3·6 %, in absence and presence of NaF respectively; in another experiment (Table XV) inorganic P 39·3 and 27 %, hexosediphosphate-P 0 and 19·8 % in absence and presence of NaF. The authors' comment on these figures is that it is "at once noticeable that no accumulation of the 'hexosediphosphate' fractions has taken place...but a piling up of inorganic phosphate" at the expense of phosphagen, adenylpyrophosphate, hexosediphosphate and insoluble "resistant esters". It is true that the accumulation was only relative, but if a relative accumulation amounting to 10 or even 20 % of the total acid-soluble P was insignificant, the technique was clearly inadequate for the problem.

In the interpretation of other experiments which have been adduced as evidence of non-phosphorylating glycolysis—the effect of dialysis or NaF on the glycolysis of tissue slices or brei, or the substrate-selectivity displayed by these enzyme systems—the limitations imposed by the presence of the cell membrane or by the location of the enzyme in the residual cell débris have sometimes been given insufficient weight. It is well known that the ease with which a diffusible constituent may be removed from a tissue brei or even from a cell-free extract is very variable. Thus, by dialysis against water, cozymase may be readily removed from yeast preparations but less readily from muscle extract, while for the removal of cocarboxylase alkaline washing is necessary. It cannot be assumed that because, for example, glutathione is removed by dialysis under arbitrary conditions other coenzymes which are ordinarily diffusible are also removed. The lack of response to added coenzymes in such cases may be due simply to the fact that the tissues still contain the optimum supply. On the other hand if the coenzymes have all been removed by dialysis it is necessary to restore them all to obtain reactivation; in the experiments of Needham & Lehmann [1937, p. 1230] on reactivation of dialysed chick embryo brei, for instance, in no case were adenylpyrophosphate, cozymase and Mg^{++} added simultaneously.

The difficulty in removing even inorganic P from tissue brei is illustrated in the following experiment. Weighed samples of about 1·0 g. rabbit brain were ground in a mortar with 0·5 g. silver sand and transferred quantitatively to centrifuge pots with 6 ml. borate buffer, two samples at *p*H 6·0 and two at *p*H 8·4; 2 ml. *M* $CaCl_2$ were added with stirring to each pot and after 20 min. at room temperature the residue was spun down and washed with the same buffer as before. All the residues were then separately extracted three times with borate buffer at *p*H 6·0 and finally twice with 5 ml. 5 % trichloroacetic acid; the inorganic P in all the extracts was estimated. The results in Table VI show that only 80–85 % of the total inorganic P present in the tissue could be extracted by buffer at *p*H 6·0, whether or not it had been previously rendered insoluble. It is not improbable that a residual cell structure which can prevent the outward diffusion of P can also prevent the inward diffusion of Ca. For this reason previous experiments on the immobilization of inorganic P by Ca salts in alkaline solution [e.g. Ashford & Holmes, 1929] do not give very satisfactory evidence on the participation of inorganic P in glycolysis, and moreover do not exclude the esterification of sugar by transference of P from adenylpyrophosphate.

There is other reason to suppose that there is an intracellular structure (perhaps for instance a lipoprotein complex) regulating the diffusion of sub-

Table VI. *Extraction of inorganic P from brain tissue*

	A	B	C	D
g. tissue	0·87	0·93	0·92	0·84
	mg. P/g. tissue			
Extracts 1+2, *p*H 8·4	0·024	0·014	—	—
Extracts 1+2, *p*H 6·0	—	—	0·198	0·198
Extract 3, *p*H 6·0	0·168	0·192	0·025	0·027
Extract 4, *p*H 6·0	0·058	0·054	<0·005	<0·005
Extract 5, *p*H 6·0	<0·005	<0·005	<0·005	<0·005
Extract 6, trichloroacetic acid	0·049	0·046	0·052	0·055
Extract 7, trichloroacetic acid	<0·005	<0·005	<0·005	<0·005
Total inorganic P extracted	0·299	0·306	0·275	0·280
% extracted in buffer	84	85	81	81

stances throughout the cytoplasm which may persist in cell fragments even if the outer membrane is disrupted. It is known for instance that if toluene is added to fresh yeast, the autofermentation rate is greatly increased [Harden, 1911]; it is difficult to account for this fact without postulating an internal change in the accessibility of the enzyme to the glycogen. It is clear that in cases where a glycolytic enzyme has not been obtained in a cell-free extract the existence of an intracellular structure affecting the accessibility of substrates has not in fact been excluded. For these reasons the view of Lehmann & Needham [1938] that "substrate-preference experiments cannot be interpreted as due to selective penetration of cell-membranes" is hardly justified till they have obtained from chick embryo a cell-free glycolysing extract which shows the same substrate-selectivity as the enzyme in the cell or cell-brei. It has been shown by Euler *et al.* [1936] and Boyland & Boyland [1938] that in brain and tumour, the selectivity exhibited by intact tissue or tissue slices to fructose and glycogen is not a property of the cell-free extract. The latter authors have pointed out that the differential inhibition of the reaction phosphoglyceric → phosphopyruvic acid observed in brei at concentrations of NaF which do not inhibit glycolysis is probably due to the accessibility of the enzyme exposed on the injured surface both to added phosphoglyceric acid and to fluoride. The behaviour of chopped brain tissue towards glycogen, glucose and hexosediphosphate [Ashford, 1934; Ashford & Holmes, 1929] is also explicable on this basis, glucose being broken down rapidly within the cell fragment, glycogen and hexosediphosphate by enzymes exposed on the surface and partly inactivated by loss of coenzymes.

Apart from this type of experiment the most important evidence put forward in support of the hypothesis of a non-phosphorylating glycolysis is the specific inhibition of glycolysis in tumour, chick embryo etc. by *l*-glyceraldehyde. It has been shown that the phosphorylating glycolysis of dialysed muscle extracts and of washed dried yeast is also inhibited by glyceraldehyde [Adler *et al.* 1937; Boyland & Boyland, 1938]. These findings have been criticized by Lehmann & Needham [1938] on the ground that the concentration necessary for inhibition was so large that the effect is probably not the specific effect observed in tumour etc. They find it also difficult to see how the normal glycolysis of the embryo can involve the formation of hexosediphosphate, since the latter should give rise to dihydroxyacetonephosphate, and the inhibiting glyceraldehyde should in that case be removed by condensation to ketosemonophosphate. The following hypothesis explains this discrepancy and is based on the assumption that the glycolytic cycle in chick embryo is essentially similar to that of living yeast. It is known that the enzymic condensation of *d*- or *l*-glyceraldehyde with dihydroxy-acetonephosphate leads to the irreversible formation of 1-fructose- and 1-sorbose-

phosphates respectively; these esters do not give rise to the equilibrium mixture of hexose-6-phosphates [Meyerhof *et al.* 1936] and take no further part in the glycolysis. Since glycolysis is inhibited by *l*- and not by *d*-glyceraldehyde, it seems probable that the fixation of triosephosphate, which can take place with either isomeride, is not the point of primary inhibition; the actual point is immaterial to the argument. The concentration of glyceraldehyde necessary for inhibition in a particular case will however be determined by the amount of triosephosphate—practically speaking by the amount of hexosediphosphate—available for fixation of the inhibitor. In the intact chick embryo the amount of hexosediphosphate present at one time will be very small; if the added glyceraldehyde can trap all the triosephosphate formed, then the glycolytic cycle will be immobilized since the further formation of adenylpyrophosphate and consequently of hexosediphosphate will be suppressed, the primary inhibition being reinforced by a secondary blockage. If however some proportion of the triosephosphate escapes fixation, the glycolytic cycle can proceed, even if diminished in extent or slowed in rate, until eventually the glyceraldehyde is decreased, by condensation, below the inhibitory concentration. The inhibition by glyceraldehyde in low concentrations is in fact transitory; with $M/800$ in chick embryo the duration was about 1 hr.

On the other hand, both dried yeast and yeast juice normally contain hexosediphosphate; if none is present, and in the absence of adenylpyrophosphate, it is necessary to add hexosediphosphate or triosephosphate to initiate glycolysis. Moreover, yeast and muscle preparations have a relatively high esterifying power (2 g. dried yeast for instance can esterify 31 mg. P in about 10 min.) and the esterified P, mainly hexosediphosphate, remains in this type of fermentation as a by-product; it can be utilized for the removal of glyceraldehyde without affecting the subsequent fermentation. The concentration of glyceraldehyde necessary for inhibition will therefore be much higher than in a tissue of low glycolytic activity, in which a limited amount of P is taking part in a cycle.

It is premature to stress too much the lack of positive evidence of a non-phosphorylating glycolysis, for instance the extraction of an enzyme which does not require the presence of cozymase etc., since the history of the preparation of similar extracts is one of frequent trials and many failures. Nevertheless the evidence is at present negative and is based chiefly on the failure to demonstrate phenomena which are characteristic of the glycolysis of cell-free extracts rather than of cells. A critical examination of the evidence shows that in at least two of the tissues discussed, brain and retina, esterification of P during glycolysis can be inferred from the experimental data; in other cases, e.g. the inhibition by glyceraldehyde and the behaviour of tissue brei, the facts can be as consistently explained on the basis of a phosphorylating glycolysis as on any other basis. It appears that the existence of a glycolytic enzyme of a non-phosphorylating type has not yet been experimentally established.

Summary

1. In the normal "resting" yeast cell hexosediphosphate is present in small amounts, approximately 1 mg. P/100 g. yeast.

2. During the fermentation of hexoses by living yeast the organic P present in the yeast is increased by about 30 mg. P/100 g. yeast, of which at least 10 mg. P are hexosediphosphate. This ester was identified and estimated by the formation of the crystalline phenylhydrazine salt of 6-phosphoglucosazone.

3. When the fermentation of sugars was inhibited by the addition of NaF, phosphoglyceric acid in amount equal to 7 mg. P/100 g. was isolated from the fresh yeast.

4. The observed changes in the distribution of P during fermentation in the living cell are shown to be in accord with the Embden-Meyerhof theory of alcoholic fermentation in cell-free extracts.

5. The evidence which has been put forward in favour of a theory of non-phosphorylating glycolysis in certain tissues is discussed. It is concluded that this theory is not yet experimentally justified.

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LXXI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS

LXI. THE MOLECULAR CONSTITUTION OF GEODIN AND ERDIN, TWO CHLORINE-CONTAINING METABOLIC PRODUCTS OF *ASPERGILLUS TERREUS* THOM. PART II. DIHYDROGEODIN AND DIHYDROERDIN AND THE SYNTHESIS OF THEIR TRIMETHYL ETHERS

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Raistrick & Smith [1936] showed that a strain of *Aspergillus terreus* Thom, when grown on Czapek-Dox solution containing glucose as sole source of carbon and KCl as sole source of chlorine, produced two new metabolic products, geodin, $C_{15}H_6O_5Cl_2(OCH_3)_2$, and erdin, $C_{15}H_7O_6Cl_2(OCH_3)$, each of which on catalytic reduction gave rise to a dihydro-derivative. Later, Clutterbuck *et al.* [1937] found that both dihydrogeodin and dihydroerdin yielded the same compound on complete methylation with diazomethane, viz. a neutral substance

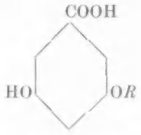
$$C_{15}H_5O_2Cl_2(OCH_3)_5,$$

which on alkaline hydrolysis yielded a monobasic acid $C_{15}H_6O_3Cl_2(OCH_3)_4$, one methoxyl group having been lost. Since, as is shown later by synthesis, the latter compound contains a COOH group the formula of the neutral, fully methylated substance, may be expanded to $C_{14}H_5OCl_2(OCH_3)_4(COOCH_3)$ and the following experiment showed that this substance is undoubtedly dihydrogeodin trimethyl ether, the $COOCH_3$ group being already present in dihydrogeodin itself but absent from dihydroerdin. When dihydrogeodin and dihydroerdin are each fully ethylated with diazoethane and the resulting neutral compounds hydrolysed by aqueous alcoholic NaOH, the same monobasic acid is obtained in each case, viz. $C_{14}H_5OCl_2(OCH_3)(OC_2H_5)_3(COOH)$, which still retains the methoxyl group originally present in dihydroerdin. This substance is therefore dihydroerdin triethyl ether. The corresponding monobasic acid obtained by hydrolysis of fully methylated dihydrogeodin or dihydroerdin is therefore dihydroerdin trimethyl ether and a further consequence is that dihydrogeodin is the methyl ester of dihydroerdin.

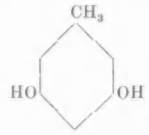
Much light has been thrown on the detailed structure of dihydrogeodin and dihydroerdin by a study of the products of reductive and hydrolytic fission.

(*a*) *Reductive fission.* When dihydrogeodin was refluxed with hydriodic acid (*d*, 1·7) two molecules of methyl iodide and one of CO_2 were evolved, and the

only non-volatile degradation products found were 3:5-dihydroxybenzoic acid (α-resorcylic acid) (I; $R=H$; $C_7H_6O_4$) and orcinol (II; $C_7H_8O_2$).



(I)



(II)

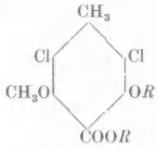
Since it is very unlikely that both these substances can arise from the same half of the molecule under the conditions of the experiment it follows that all the C atoms in dihydrogeodin are accounted for, and the action of HI may be summarized as $C_{17}H_{14}O_7Cl_2+3H_2O+4H=C_7H_8O_2+C_7H_6O_4+2CH_3OH$ (as $2CH_3I$) $+CO_2+2HCl$.

Dihydroerdin also yielded the same products (but with 1 mol. CH_3I in place of 2 mol.) and it is clear that the basal structure of each dihydro-compound consists of the orcinol nucleus linked in some way to the α-resorcylic acid nucleus. A consideration of the expanded formula $C_{14}H_5OCl_2(OCH_3)_4(COOCH_3)$ previously given for dihydrogeodin trimethyl ether reveals that only one oxygen atom is unaccounted for and it seemed probable therefore that this O atom is concerned in the link between the two nuclei. An obvious possibility is —CO— and this possibility was confirmed by hydrolytic fission.

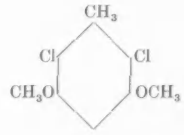
(*b*) *Hydrolytic fission.* Like many hydroxy-derivatives of benzophenone which undergo hydrolytic fission when heated with 80% H_2SO_4 [cf. Graebe & Eichengrun, 1892] both dihydrogeodin and dihydroerdin are split by this reagent and give rise to the monomethyl ether of 3:5-dihydroxybenzoic acid (α-resorcylic acid) (I; $R=CH_3$; $C_8H_8O_4$) together with an acid $C_8H_6O_4Cl_2$, a fission which may be represented thus for dihydroerdin:

$$C_{16}H_{12}O_7Cl_2+H_2O=C_8H_8O_4+C_8H_6O_4Cl_2.$$

The chlorine-containing acid was obviously a derivative of orcinol and seemed to be a dichloro-derivative of either orsellinic or *p*-orsellinic acid. On complete methylation with diazomethane it yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate (III; $R=CH_3$), identical in all respects with the compound prepared synthetically by the chlorination of methyl *p*-orsellinate dimethyl ether [Calam & Oxford, 1939]. The acid $C_8H_6O_4Cl_2$ is therefore *2:6-dichloro-3:5-dihydroxy*-p-*toluic acid* (III; $R=H$; $OCH_3=OH$).



(III)



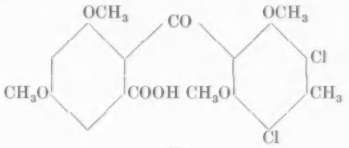
(IV)

Dihydroerdin trimethyl ether also underwent a similar hydrolytic fission to yield 3:5-dimethoxybenzoic acid, CO_2, 2:6-dichloro-orcinol dimethyl ether (IV) and the monomethyl ether of dichloro-*p*-orsellinic acid (III; $R=H$).

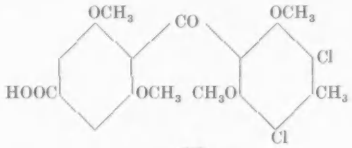
Compound (IV) was also obtained in quite another way. The thermal degradation of geodin and erdin, as well as of dihydroerdin, at 250°, yielded a sublimate consisting essentially of a phenolic substance $C_7H_6O_2Cl_2$, *2:6-dichloro-orcinol*, which on methylation yielded IV ($C_9H_{10}O_2Cl_2$) identical in all respects

with the dimethyl ether of 2:6-dichloro-orcinol prepared synthetically [Calam & Oxford, 1939].

The above evidence strongly suggests that dihydrogeodin and dihydroerdin are derivatives of benzophenone and, since two COOH groups appear in the fission products, one must be concerned in the union between the two benzene rings, dihydroerdin trimethyl ether being only a monobasic acid. The only possible links between the two rings are therefore —CO— and —CO.CH_2—. The latter must be incorrect since dihydroerdin trimethyl ether, when oxidized by boiling alkaline $KMnO_4$, forms a dibasic acid with the same number of C atoms but with two O atoms more and two H atoms less than the starting material. This can be explained only by the oxidation of CH_3 to COOH, hence the CH_3 group of the dichloro-orcinol half of the molecule must exist as such in the dihydro-compounds. The only possible link between the two benzene nuclei is therefore —CO—, and the failure of all our attempts to prepare derivatives of this carbonyl group is doubtless due to the steric effects of the adjacent OCH_3 groups. There are thus two possible structures for dihydroerdin trimethyl ether, viz.:



(V)

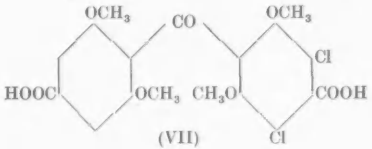


(VI)

for the free COOH group in this compound must be attached to the non-chlorinated benzene ring, there being no free position in the other ring. Each of these structures will yield 3:5-dimethoxybenzoic acid and 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid or its degradation products on hydrolytic fission. Since no dimethoxy-phthalic or -terephthalic acid is formed in the fission, the evidence so far presented does not enable a decision to be made between (V) and (VI). Actually (V) is almost certainly correct for the following reasons.

(1) Dihydroerdin trimethyl ether can be acetylated to yield a neutral monoacetyl derivative. This may be explained on (V) (a γ-keto-acid behaving as a γ-hydroxy-γ-lactone) for which there are many analogies including that of benzophenone-2-carboxylic acid [von Pechmann, 1881; Graebe, 1900], but cannot be explained convincingly on (VI).

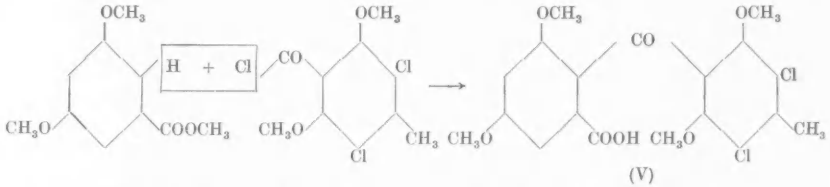
(2) The dibasic acid obtained by alkaline $KMnO_4$ oxidation of dihydroerdin trimethyl ether must be (VII) if derived from (VI). But such a structure is



(VII)

quite symmetrical about the —CO— link (except for the two Cl atoms, whose effect on the reactivity of the molecule is probably very small in comparison with that of the four methoxyl groups), and ought to yield both 3:5-dimethoxybenzoic acid and 2:6-dimethoxyterephthalic acid on hydrolytic fission. Actually 3:5-dimethoxybenzoic acid and 3:5-dichloro-2:6-dimethoxyterephthalic acid were the only hydrolytic products isolated.

(3) Dihydroerdin trimethyl ether has been synthesized by an application of the Friedel-Crafts reaction, 2:6-dichloro-3:5-dimethoxy-*p*-toluyl chloride being condensed directly with methyl 3:5-dimethoxybenzoate. From the known



behaviour of resorcinol dimethyl ether towards acid chlorides in the Friedel-Crafts reaction it might be predicted with confidence that in the above more complex instance the entering group would also occupy the position *para* to one OCH_3 group and *ortho* to the other, to the almost total exclusion of the position *ortho* to each OCH_3 group. The presence of $COOCH_3$ *meta* to each OCH_3 would tend to strengthen this preference. Thus Jones & Robinson [1917], discussing the influence of a negative group on the directing effect of a positive group in benzene substitution, conclude "there is evidence that it is not merely *ortho* substitution which is favoured but that it is the particular *ortho* position situated between the positive and negative groups". There can be little doubt therefore that dihydroerdin trimethyl ether is *3′:5′-dichloro-4:6:2′:6′-tetramethoxy-4′-methylbenzophenone-2-carboxylic acid* (V). Since the hydrolytic fission of dihydroerdin itself yields the monomethyl ether of α-resorcylic acid, dihydroerdin is evidently either 3′:5′-dichloro-6:2′:6′-trihydroxy-4-methoxy- or 3′:5′-dichloro-4:2′:6′-trihydroxy-6-methoxy-4′-methylbenzophenone-2-carboxylic acid, but the evidence so far adduced offers no clue as to which structure is the correct one.

The methylation of synthetic dihydroerdin trimethyl ether with diazomethane yielded its methyl ester identical in all respects with dihydrogeodin trimethyl ether obtained by the complete methylation of dihydrogeodin, prepared by the catalytic reduction of geodin from *A. terreus*. Dihydrogeodin trimethyl ether is thus *methyl 3′:5′-dichloro-4:6:2′:6′-tetramethoxy-4′-methyl-benzophenone-2-carboxylate* (V; $COOH = COOCH_3$).

Experimental

Equivalents of dihydrogeodin and dihydroerdin. The compounds were dissolved in aqueous ethanol and titrated directly with 0·1 *N* NaOH, with phenolphthalein as indicator. *Dihydrogeodin:* Found, 322. $C_{17}H_{14}O_7Cl_2$ requires 401 (as monobasic acid) and 200·5 (as dibasic acid). *Dihydroerdin:* Found, 201. $C_{16}H_{12}O_7Cl_2$ requires 193·5 (as dibasic acid).

Dihydroerdin is thus clearly dibasic and dihydrogeodin more nearly monobasic than dibasic.

Ethylation of dihydrogeodin and dihydroerdin and subsequent hydrolysis to dihydroerdin triethyl ether

Dihydrogeodin (0·45 g.) was treated with excess of ethereal diazoethane for 30 min. The solvent was removed leaving a reddish gum (0·64 g.) which was hydrolysed by refluxing with ethanol (50 ml.) and *N* NaOH (25 ml.) for 1 hr. After removal of the ethanol *in vacuo* and acidification, the precipitated acid

was collected (0·59 g.; M.P. 185°) and crystallized from benzene-light petroleum in colourless rectangular prisms, M.P. 211–213°. (Found: C, 55·95, 56·0; H, 5·2, 5·0; CH_3O, 24·9, 25·1 %. $C_{22}H_{24}O_7Cl_2$ (dihydroerdin triethyl ether) requires C, 56·05; H, 5·1; $CH_3O+3C_2H_5O$ (calc. as $4CH_3O$), 26·3 %. $C_{21}H_{22}O_7Cl_2$ (diethyl-dihydrogeodin) requires C, 55·1; H, 4·85; $2CH_3O+2C_2H_5O$ (calc. as $4CH_3O$), 27·1 %.) Dihydroerdin, treated in exactly the same way, gave identical rectangular prisms, M.P. 211–212°, alone or mixed with the acid obtained from dihydrogeodin. (Found: C, 56·2; H, 5·1; Cl, 14·8, 14·7; CH_3O, 25·1, 25·5 %. Equiv. 486. $C_{22}H_{24}O_7Cl_2$ requires C, 56·05; H, 5·1; Cl, 15·05; $CH_3O+3C_2H_5O$ (calc. as $4CH_3O$), 26·3 %. Equiv. 471.)

Acetyl derivative of dihydroerdin trimethyl ether

Dihydroerdin trimethyl ether (0·5 g.) was heated for 30 min. at 150–160° with anhydrous sodium acetate (1 g.), acetic anhydride (2 ml.) and glacial acetic acid (3 ml.). Water was then cautiously added to the cooled mixture, and the precipitated gum hardened after standing for several hours. The solid product was crystallized from light petroleum (B.P. 80–100°) and separated in rosettes of prisms (0·13 g.). Unlike the starting material it was not completely soluble in dilute $NaHCO_3$ solution. The $NaHCO_3$-insoluble portion (0·03 g.) was crystallized from CCl_4-light petroleum and formed compact square prisms, M.P. 208–210°. (Found: C, 53·5, 53·5; H, 4·15, 4·35; Cl, 15·1; hydrolysable $CH_3.CO$, 8·6; CH_3O, 24·85 %. $C_{21}H_{20}O_8Cl_2$ requires C, 53·5; H, 4·3; Cl, 15·05; $CH_3.CO$, 9·1; $4CH_3O$, 26·3 %.) The acetyl derivative is quite insoluble in cold caustic soda solution and dissolves only slowly on heating.

Hydrolysis. 0·02382 g. in 10 ml. EtOH + 5 ml. H_2O required only 0·004 ml. $N/10$ NaOH for neutralization to phenolphthalein. 1·891 ml. $N/10$ NaOH were then added and the corked flask kept at 37° overnight. Required: 1·034 ml. $N/10$ H_2SO_4 for back titration. Hence equiv. = 278. $C_{21}H_{20}O_8Cl_2$ titrating as a dibasic acid requires equiv. = 235·5.

Oxidation of dihydroerdin trimethyl ether to a dibasic acid containing the same number of carbon atoms

Dihydroerdin trimethyl ether, $C_{19}H_{18}O_7Cl_2$ (0·5 g.), was dissolved in 0·1 N NaOH (30 ml.) and the solution refluxed with slow dropwise addition of 5 % aqueous $KMnO_4$. A stable purple colour was reached after 30 ml. of the latter had been added during $2\frac{1}{2}$ hr. The liquid was cooled, decolored with SO_2 and acidified with dilute H_2SO_4. The precipitated acid separated as colourless needles (0·33 g., M.P. 229–231°), raised to 233–235° by recrystallization from hot water. (Found: C, 49·85; H, 3·6; Cl, 15·7, 15·25; CH_3O, 27·3 %. Equiv. 227. $C_{19}H_{16}O_9Cl_2$ requires C, 49·7; H, 3·5; Cl, 15·45; $4CH_3O$, 27·0 %. Equiv. 229·5 (titrating as a dibasic acid).)

Several attempts were made partially to methylate dihydroerdin with diazomethane and then to oxidize away the incompletely methylated parts of the molecule in the hope of isolating a recognizable dimethoxy-phthalic or -terephthalic acid, but even the product obtained by the addition of 2 mol. of diazomethane to dihydrogeodin was completely destroyed by $KMnO_4$ both in aqueous alkaline and in acetone solutions.

Reductive fission experiments

A. *Dihydrogeodin.* Dihydrogeodin (0·8487 g.) was heated with HI (15 ml.; d, 1·7) in a Zeisel apparatus in a stream of dry CO_2-free nitrogen, the bath temperature being maintained at 140–150°. The issuing gases were passed

through bubblers containing alcoholic $AgNO_3$ and standard baryta solution respectively. The AgI produced in the former amounted to 0·9739 g. (calc. for $2CH_3O$, 0·9928 g.) whilst the CO_2 absorbed in the latter was equivalent to 44·05 ml. 0·1 *N* (calc. for 1 mol. CO_2, 42·3 ml. 0·1 *N*). The residual HI solution was washed into a continuous extractor and extracted with ether for 8 hr. The material left after removal of the solvent was dissolved in water and heated on the steam bath with red phosphorus to remove iodine, filtered and re-extracted with ether. The dried solid obtained from the final extract (0·635 g.; calc. 0·627 g.) was fractionally sublimed in a high vacuum, first at 80° to yield sublimate I (0·22 g.) and then at 160° to yield sublimate II (0·25 g.).

Sublimate I, after re-sublimation, was free from chlorine and melted at 108° alone or mixed with authentic anhydrous orcinol (M.P. 108°). In aqueous alcoholic solution it gave a violet colour with $FeCl_3$, identical with that given by orcinol. (Found: C, 68·0, 67·7; H, 6·5, 6·4 %. $C_7H_8O_2$ requires C, 67·7; H, 6·5 %.)

Sublimate II, after re-sublimation, was free from chlorine and melted at 236°, alone or mixed with authentic α-resorcylic acid (M.P. 236°) obtained by the HI demethylation of 3:5-dimethoxybenzoic acid. (Found: C, 54·8, 54·55; H, 4·1, 3·9 %. $C_7H_6O_4$ requires C, 54·5; H, 3·9 %.) On methylation with diazomethane and hydrolysis of the resulting methyl ester, 3:5-dimethoxybenzoic acid was obtained, M.P. 181–182°, alone or mixed with the authentic acid prepared by the hot alkaline $KMnO_4$ oxidation of orcinol dimethyl ether.

B. *Dihydroerdin.* Dihydroerdin (0·873 g.) under the same conditions gave CO_2 equivalent to 44·5 ml. 0·1 *N* (calc. for 1 mol. CO_2, 45·5 ml.) and CH_3I (as AgI) equivalent to one CH_3O group. From the HI was recovered 0·68 g. of crude solid material (calc. 0·64 g.) which was fractionally sublimed as before to yield orcinol (0·15 g.) and a small amount of α-resorcylic acid.

Hydrolytic fission experiments

A. *Dihydrogeodin.* Dihydrogeodin (1 g.) was gradually heated in a small flask on a wire gauze with a mixture of conc. H_2SO_4 (20 ml.) and water (10 ml.) with a thermometer in the liquid. At 120° the colour of the liquid suddenly became lighter, the heating was immediately stopped and the flask allowed to cool. Water (100 ml.) was then cautiously added and the precipitated solid collected, washed and dried (0·5 g.). The filtrate, after ether extraction, yielded a similar solid (0·5 g.). The combined products were repeatedly and fractionally sublimed in a high vacuum giving two main fractions.

Sublimate I sublimed at 110–120°, melted at 214° and gave an intense blue colour with $FeCl_3$. (Found: C, 40·7, 40·6; H, 2·7, 2·8; Cl, 29·4, 29·6 %. $C_8H_6O_4Cl_2$ requires C, 40·5; H, 2·55; Cl, 30·0 %.) It was shown to be *2:6-dichloro-3:5-dihydroxy-*p*-toluic acid* by complete methylation with diazomethane when methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate, M.P. 85–86°, was obtained, identical in all respects (crystalline form; mixed M.P.; solubilities) with the ester prepared synthetically [Calam & Oxford, 1939]. (Found: C, 47·7; H, 4·3; Cl, 25·2, 25·2; CH_3O, 33·7 %. $C_{11}H_{12}O_4Cl_2$ requires C, 47·3; H, 4·3; Cl, 25·4; $3CH_3O$, 33·3 %.) The above fission product, when heated above its M.P., lost CO_2 to yield *2:6-dichloro-orcinol*, M.P. 164°, identical with the chlorophenol obtained by thermal degradation of erdin (see later).

Sublimate II sublimed at 120–140°, and was finally obtained pure only after repeated fractional sublimation. It melted at 201° and did not contain chlorine, nor did it give any coloration with $FeCl_3$. It was soluble in $NaHCO_3$ solution with evolution of CO_2. (Found: C, 56·8; H, 4·9; CH_3O, 17·2 %. $C_8H_8O_4$ requires

C, 57·15; H, 4·8; $1CH_3O$, 18·4 %.) It was shown to be the monomethyl ether of α-resorcylic acid (Mauthner [1927] gives M.P. 202–203°) by methylating it with diazomethane followed by hydrolysis of the resulting ester with aqueous alcoholic NaOH to yield 3:5-dimethoxybenzoic acid, M.P. 181–182°, alone or mixed with an authentic specimen.

B. *Dihydroerdin.* A more efficient and rapid method of separation of the fission products was devised in this case, advantage being taken of the sparing solubility of the sodium salt of the monomethyl ether of α-resorcylic acid in an excess of $NaHCO_3$ solution. Dihydroerdin (1·207 g.) and a mixture of conc. H_2SO_4 (22 ml.) and water (11 ml.) were heated together in a stream of CO_2-free nitrogen and the issuing gases passed through a bubbler containing standard baryta solution. The reaction flask was slowly heated in an oil bath and when the latter had reached 125° the red solution suddenly became nearly colourless, with simultaneous separation of a solid. The oil bath was at once removed and the reaction mixture cooled. The CO_2 evolved was equivalent to only 3·5 ml. 0·1 *N* NaOH. Water (65 ml.) was added to the reaction mixture and the precipitated solid was later collected, washed and dried. The crude product (1·02 g.) was shaken with a slight excess of saturated $NaHCO_3$ solution to neutralize it, and again filtered.

The filtrate on acidification gave an acid (0·42 g.), M.P. 200–205°, raised to 214° by a single sublimation in a high vacuum. This acid was identical with the acid, M.P. 214°, obtained in the hydrolytic fission of dihydrogeodin, and was therefore *2:6-dichloro-3:5-dihydroxy*-p-*toluic acid*. On methylation with diazomethane it yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate identical in all respects with an authentic specimen of the ester prepared synthetically.

The $NaHCO_3$-insoluble residue was treated with dilute H_2SO_4 and extracted with ether, the extract being then shaken with dilute aqueous $NaHCO_3$. The non-acidic substance remaining in the ether (0·03 g.; calc. from CO_2 produced in the fission, 0·034 g.) gave on sublimation a product of M.P. 165°, identical with *2:6-dichloro-orcinol* obtained by thermal degradation of erdin (see later). The above bicarbonate extract was acidified and extracted with ether to yield a product (0·54 g.) which, when crystallized once from hot water, had M.P. 197–197·5°, and was free from chlorine. It was identical in all respects with the monomethyl ether of α-resorcylic acid obtained in the hydrolytic fission of dihydrogeodin. (Found: C, 56·7, 56·7; H, 4·95, 4·7; CH_3O, 17·15, 17·35 %. $C_8H_8O_4$ requires C, 57·15; H, 4·8; $1CH_3O$, 18·4 %.)

C. *Dihydrogeodin trimethyl ether.* The ether (1·0 g.) was heated with a mixture of conc. H_2SO_4 (20 ml.) and water (10 ml.) to 120° when the colour suddenly changed from deep orange to light brown. After cooling, water (40 ml.) was added and the precipitated solid (1·15 g.) collected later, washed and dried. It was covered with a slight excess of saturated $NaHCO_3$ solution, and, after standing for a while, filtered.

The filtrate, on acidification, yielded a crude product (0·59 g.) which after one recrystallization from benzene gave rather impure 3:5-dimethoxybenzoic acid (0·24 g.) (see below). The benzene mother liquor, on evaporation, yielded a different substance (0·17 g.) which was purified by sublimation followed by crystallization from aqueous alcohol. It formed hair-like needles, M.P. 201°, which contained chlorine and gave a violet colour with $FeCl_3$. (Found: C, 43·1, 43·1; H, 3·2, 3·15; CH_3O, 12·2 %. $C_9H_8O_4Cl_2$ requires C, 43·0; H, 3·2; $1CH_3O$, 12·35 %.) That it was 2:6-dichloro-3-hydroxy-5-methoxy-*p*-toluic acid (III, $R = H$) was shown by its identity with this substance prepared synthetically (Calam & Oxford [1939] who quote M.P. 202–203°) and by the fact that it

yielded methyl 2:6-dichloro-3:5-dimethoxy-*p*-toluate (III; $R=CH_3$) on methylation with diazomethane.

The $NaHCO_3$-insoluble portion mentioned above was treated with 0·2 *N* NaOH (10 ml.), and the undissolved residue (0·12 g.) was recrystallized from light petroleum to yield prisms, M.P. 131–133°, not depressed on admixture with an authentic specimen of 2:6-dichloro-orcinol dimethyl ether (IV). The alkali-soluble material was recovered (0·25 g.) and proved to be chiefly 3:5-dimethoxybenzoic acid (0·49 g. in all). The combined specimens were recrystallized from hot water to yield needles, M.P. 181–182°, not depressed on admixture with an authentic specimen of 3:5-dimethoxybenzoic acid.

D. *The dibasic acid $C_{19}H_{16}O_9Cl_2$ obtained by oxidation of dihydroerdin trimethyl ether.* The acid (0·85 g.) was heated with conc. H_2SO_4 (17 ml.) and water (8 ml.) to 110° when the colour changed from orange to light brown. After cooling, an equal volume of water was added and the precipitated solid collected after an hour (0·55 g.). It gave only a faint violet coloration with $FeCl_3$.

After solution in dilute aqueous $NaHCO_3$ and filtration from a little tarry material it was reprecipitated (0·25 g.) and purified, by sublimation in a high vacuum at 100°, to yield chlorine-free 3:5-dimethoxybenzoic acid, M.P. and mixed M.P. 183°. (Found: CH_3O, 33·1 %. $C_9H_{10}O_4$ requires $2CH_3O$, 34·1 %.)

The original acid mother liquor from the fission and the filtrate from the above crude dimethoxybenzoic acid were extracted with ether and the combined crude products (0·44 g.) crystallized from ethyl acetate-light petroleum to give colourless needles, M.P. 236–238°, alone or mixed with authentic 3:5-dichloro-2:6-dimethoxyterephthalic acid, M.P. 235–237° [Calam & Oxford, 1939]. (Found: C, 40·1; H, 2·8; Cl, 24·7 %. Equiv. 143·5. $C_{10}H_8O_6Cl_2$ requires C, 40·7; H, 2·7; Cl, 24·1 %. Equiv. 147·5.)

Thermal degradation of geodin, erdin and dihydroerdin with production of 2:6-dichloro-orcinol

Geodin, erdin and dihydroerdin all give the same volatile product on thermal degradation. The following is a typical experiment: erdin (1 g.) was heated at 250° for $1\frac{1}{4}$ hr. in a long boiling tube fitted with a small condenser, when long, colourless needles sublimed (0·15 g.). This product, after resublimation, melted at 164°. (Found: C, 43·9, 44·1; H, 3·1, 3·2; Cl, 36·0, 36·3 %; mol. wt. (cryoscopic in camphor), 193, 189. $C_7H_6O_2Cl_2$ requires C, 43·5; H, 3·1; Cl, 36·75 %; mol. wt. 193.) It was soluble in caustic soda solution and contained two active H atoms as determined by the Zerewitinoff method (Roth). (Found: 1·8 active H atoms both in anisole at 19° and in pyridine at 95°.) It also contained one Me side chain, as determined by Kuhn-Roth oxidation. (Found: 0·947, 0·943 mol. of CH_3COOH per mol. of $C_7H_6O_2Cl_2$) and was therefore undoubtedly a dichlorodihydroxymethylbenzene. That it was *2:6-dichloro-orcinol* was proved by methylation with excess of ethereal diazomethane, when 2:6-dichloro-orcinol dimethyl ether (IV), M.P. 129°, was obtained, identical in all respects, including mixed M.P., with the authentic compound, M.P. 133–134°, prepared synthetically [Calam & Oxford, 1939]. (Found: C, 49·0, 48·8; H, 4·4, 4·55; Cl, 31·6, 31·6 %. $C_9H_{10}O_2Cl_2$ requires C, 48·8; H, 4·6; Cl, 32·1 %.) Dihydroerdin behaved very similarly to erdin, but with geodin the yield was smaller and the sublimate less pure.

Syntheses

A. *Dihydroerdin trimethyl ether. (3′:5′-Dichloro-4:6:2′:6′-tetramethoxy-4′-methylbenzophenone-2-carboxylic acid* (V).) A mixture of 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid (5 g.) and thionyl chloride (7 ml.) was kept for an hour,

after which excess of the latter was removed under reduced pressure. The resulting crude acid chloride, methyl 3:5-dimethoxybenzoate (5·5 g.) and powdered anhydrous aluminium chloride (5 g.), were placed in a long-necked round-bottomed flask, which, after displacement of the air by dry HCl gas, was fitted with a cork bearing a $CaCl_2$-tube. The mixture was heated on the steam bath for 10 min. until a homogeneous reddish-brown semi-solid mass had formed and was left at room temperature for 2 days. Water was added and unchanged methyl 3:5-dimethoxybenzoate removed by distillation in steam. The liquid was decanted from the residual gum which was methylated by treatment with acetone (30 ml.), methyl sulphate (10 ml.) and 2*N* NaOH (80 ml. in 8 successive portions of 10 ml.) with continuous shaking. The semi-solid product was washed by decantation several times and hydrolysed by refluxing with ethanol (50 ml.) and *N* NaOH (50 ml.) for 1 hr. After addition of water, cooling and filtration from a little oily impurity, the crude acid was precipitated by acidification (3·7 g.). After one crystallization from CCl_4-light petroleum it melted at 149–159° (1·6 g.) and from the mother liquor 1·4 g. of nearly pure 2:6-dichloro-3:5-dimethoxy-*p*-toluic acid were recovered, M.P. 107–112°. The product, M.P. 149–159°, was further purified by repeated crystallization from CCl_4 to yield colourless laminae, M.P. 177–179°, readily soluble in dilute aqueous $NaHCO_3$. (Found: C, 52·65; H, 4·1; Cl, 16·6, 16·8; CH_3O, 28·6, 28·8 %. $C_{19}H_{18}O_7Cl_2$ requires C, 53·1; H, 4·2; Cl, 16·55; $4CH_3O$, 28·9 %.) Clutterbuck *et al.* [1937] quote 168° for the M.P. of natural dihydroerdin trimethyl ether, but the M.P. of their material was raised after several recrystallizations from CCl_4 to 177–179° unaltered by admixture with the above synthetic product of the same M.P.

B. *Dihydrogeodin trimethyl ether.* (*Methyl 3′:5′-dichloro-4:6:2′:6′-tetramethoxy-4′-methylbenzophenone-2-carboxylate* (V; $COOH = COOCH_3$).) The above synthetic dihydroerdin trimethyl ether (M.P. 177–179°; 0·1 g.) was treated with excess of ethereal diazomethane, and the resulting ester crystallized from aqueous methyl alcohol to yield colourless needles, M.P. 110–112°, alone or mixed with dihydrogeodin trimethyl ether (M.P. 108°) prepared from natural dihydrogeodin. (Found: C, 53·65; H, 4·2; Cl, 16·05, 15·8; CH_3O, 34·3 %. $C_{20}H_{20}O_7Cl_2$ requires C, 54·15; H, 4·55; Cl, 16·0; $5CH_3O$, 35·0 %.)

C. *Decarboxydihydroerdin trimethyl ether.* (*3:5-Dichloro-2:6:2′:4′-tetramethoxy-4-methylbenzophenone.*) Resorcinol dimethyl ether (5 g.) and anhydrous aluminium chloride (5 g.) were added to crude 2:6-dichloro-3:5-dimethoxy-*p*-toluyl chloride (5 g.) prepared as before (see above). The mixture was cooled, the air displaced by dry HCl gas, and the flask fitted with a cork bearing a $CaCl_2$-tube. After heating on the water bath until a homogeneous dark red mass had formed, the flask was kept for 2 days. The reaction mixture was decomposed with ice-water and the unchanged resorcinol dimethyl ether removed by steam distillation. The residual solid was taken up in ether, the extract washed with dilute aqueous $NaHCO_3$, and the solvent removed to yield a crude product (4·2 g.) which was dissolved in acetone (20 ml.) and treated with dimethyl sulphate (10 ml.) and 2*N* NaOH (80 ml. in ten successive lots) with continuous shaking. The solid product (4 g.) was crystallized from aqueous methyl alcohol to yield colourless parallelepipeds, M.P. 94–95°. (Found: C, 56·4, 56·3; H, 4·8, 4·7; Cl, 18·4; CH_3O, 31·5 %. $C_{18}H_{18}O_5Cl_2$ requires C, 56·1; H, 4·7; Cl, 18·4; $4CH_3O$, 32·2 %.)

Many attempts were made to decarboxylate dihydroerdin trimethyl ether in the hope of obtaining the above substituted benzophenone. These included heating in a sealed tube with water at 140° and with water and Cu bronze at

250°, heating the ether in the dry state in N_2 at 250–300° (practically no CO_2 evolved) and heating with quinoline and Cu chromite. All were fruitless, the material being either completely broken down or recovered unchanged.

D. *Homodecarboxydihydroerdin trimethyl ether.* (*3:5-Dichloro-2:6:2′:4′-tetramethoxy-4:6′-dimethylbenzophenone.*) The synthesis was carried out exactly as in the previous section save that orcinol dimethyl ether (5 g.) was used in place of resorcinol dimethyl ether. The crude product after methylation (3·5 g.) was purified by repeated crystallization from aqueous methyl alcohol. Large, colourless rectangular prisms, M.P. 105–106°. (Found: C, 57·1, 57·2; H, 5·1, 5·0; Cl, 17·95; CH_3O, 31·3, 31·15 %. $C_{19}H_{20}O_5Cl_2$ requires C, 57·1; H, 5·05; Cl, 17·8; $4CH_3O$, 31·1 %.)

In this preparation the $NaHCO_3$ extract of the ethereal solution of the crude product (after removal of unchanged orcinol dimethyl ether) yielded *2:6-dichloro-3:5-dihydroxy*-p-*toluic acid* on acidification; M.P. 214° after two sublimations in a high vacuum. This constitutes a synthesis of the chloro-acid obtained by the hydrolytic fission of dihydrogeodin and dihydroerdin (pp. 584–5).

Several attempts were made to oxidize both CH_3 groups in the above substituted benzophenone to COOH in the hope of obtaining the dibasic acid previously obtained by the oxidation of dihydroerdin trimethyl ether (p. 583). These included the action of $KMnO_4$ in alkaline and in acid solution; CrO_3 in acetic acid-acetic anhydride solution; $K_2Cr_2O_7$ in H_2SO_4 solution; and 25 % aqueous HNO_3. All were fruitless, the compound being either completely destroyed or recovered unchanged.

Summary

Geodin ($C_{15}H_6O_5Cl_2(OCH_3)_2$) and erdin ($C_{15}H_7O_6Cl_2(OCH_3)$), metabolic products of *Aspergillus terreus* Thom, each give rise to a dihydro-derivative on catalytic reduction, dihydrogeodin being the methyl ester of dihydroerdin. As a result of analytical and synthetic methods dihydrogeodin trimethyl ether, $C_{14}H_5OCl_2(OCH_3)_4COOCH_3$, has been shown to be *methyl 3′:5′-dichloro-4:6:2′:6′-tetramethoxy-4′-methylbenzophenone-2-carboxylate* and dihydroerdin trimethyl ether, $C_{14}H_5OCl_2(OCH_3)_4COOH$, to be *3′:5′-dichloro-4:6:2′:6′-tetramethoxy-4′-methylbenzophenone-2-carboxylic acid.*

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LXXII. CRYSTALLINE ESTERS OF VITAMIN A

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PART I

The preparation of the crystalline 2-naphthoate and anthraquinone-2-carboxylate of vitamin A has been reported by Hamano [1935; 1937] who used as starting material the non-saponifiable matter from fish liver oils which are exceptionally rich in this vitamin. Hamano found that these esters were biologically active but did not submit them to a quantitative animal assay. The work described in this paper was undertaken in order to find out whether crystalline esters could be prepared from vitamin A obtained from commercial oils and to compare their biological activity with that of the International Standard.

By molecular distillation of vitamin A alcohol, fractions with coefficients of absorption ($E^{1\%}_{1\,cm.}$ 328 mμ) exceeding 1600 were obtained. These were used for the preparation of several synthetic esters of which the 2-naphthoate and anthraquinone-2-carboxylate alone were obtained crystalline. The properties of the crystalline esters made in this way resembled those of the specimens prepared by Hamano, the anthraquinone-2-carboxylate being obtained in both the yellow and the red forms described by this author.

The ultraviolet absorption of the esters has been examined and from the results it has been deduced that the vitamin A alcohol combined in these substances has $E^{1\%}_{1\,cm.}$ 328 mμ = 1800 approximately. Preliminary biological tests on the crystalline esters have been carried out by Dr Katharine Coward at the laboratories of the Pharmaceutical Society of Great Britain and by Dr S. W. F. Underhill in the physiological laboratories of the British Drug Houses Ltd. Both substances were found to be highly active and to have similar potencies when these were calculated on the basis of vitamin A content.

The acetate, diphenylacetate, 4-nitrobenzoate and stearate of vitamin A have also been prepared but have not as yet been obtained crystalline.

It was found that distilled vitamin A alcohol having $E^{1\%}_{1\,cm.}$ 328 mμ = 1600[1] could be crystallized from methyl alcohol at low temperatures. The product obtained in this way had properties identical with those of crystalline vitamin A which had been prepared by the method of Holmes and Corbet [1937]. When working with our raw materials, distillation, followed by direct crystallization, proved to be the less tedious procedure. Two batches prepared by the latter method had $E^{1\%}_{1\,cm}$ 328 mμ = 1800 in ethyl alcohol and Carr-Price values 92,000 and 94,000.

The stability of the synthetic esters is of importance in connexion with the possibility of their use as biological standards. All the highly purified preparations of vitamin A and its esters which are described in this paper appear to deteriorate on storage in the absence of oxygen. The progress of this change is marked by a decrease in $E^{1\%}_{1\,cm.}$ 328 mμ and in Carr-Price value and the rate at which it takes place varies considerably; the crystalline 2-naphthoate and anthraquinone-2-carboxylate were found to be the most stable preparations of high potency, although the specimens of which portions were used for the biological tests deteriorated slightly during three months' storage *in vacuo*; solutions of these esters in arachis oil, however, appear to be quite stable. Crystalline vitamin A alcohol (on the other hand) appears to be more labile: Holmes and Corbet observed that the ultraviolet absorption of solutions of the crystals decreased very rapidly and a similar falling off was observed by us with a crystalline specimen during storage *in vacuo* at a temperature below its melting point.

Experimental

The purification of vitamin A by molecular distillation

Concentrates of vitamin A, substantially free from sterols, were prepared from fish liver oils in the usual manner. These were further purified by repeated fractional molecular distillation in a cyclic still of the type described by Hickman [1937]. In this apparatus, the distilland may be re-circulated through the still for any desired number of times. The rate of circulation may be varied at will, but a speed of about 6 ml. per min. was found to give the best results. Each passage of the distilland over the hot distillation surface at a temperature which may be predetermined constitutes a cycle. The distillation surface, consisting of a vertical metal cylinder, is placed co-axially within an air-cooled glass tube which acts as the condenser. The space between the hot surface and the condenser is about 1 cm. wide, and is evacuated to 1–3μ by a condensation pump with a high pumping speed. The distillate flows down the condenser into a receiver which may be changed without interrupting the distillation. In using this apparatus for the distillation of viscous vitamin A concentrates, the latter were diluted with "residue oil" which was prepared from cod liver oil by removal of material volatilizing at or below 250° in a molecular still. Many distillations

[1] Smith *et al.* [1938] and Gillam & El Ridi [1938] have shown that the ultraviolet absorption of vitamin A preparations varies with the solvent used for the determination, while Holmes & Corbet have reported that Darby noticed a rapid decrease in the extinction coefficient of solutions of the crystalline vitamin. It is on account of these observations that we give the following particulars of the determination of the extinction coefficients quoted in this paper. Pure *iso*propyl alcohol was used as solvent in all cases except for the measurements on crystalline A when ethyl alcohol was employed. The determinations were made within 20–30 min. of the preparation of the solution and the latter was subjected for about 4 min. to the action of the light from a spark between electrodes of tungsten steel.

and redistillations were carried out during the preparation of the highly purified vitamin used in the experiments described in this paper. The results of two distillations are given in Table I.

Table I. *Distillation of crude vitamin A concentrate*

Fraction	Temperature °C.	Pressure μ	No. of cycles	Wt. g.	Carr-Price value	Recovery %
Distilland: Run 7. Vitamin A concentrate, Carr-Price value 40,000					80 g.	
"Residue oil"					120 g.	
1	95	4–7	5	6·2	64,000	12
2	110	1–3	5	20·7	66,000	42
3	120	1–2	4	14·8	62,500	29
4	130	1–2	2	6·7	56,000	11
					Total recovery	94
Run 20. Mixed fractions from previous distillations, Carr-Price value 53,750					77 g.	
"Residue oil"					20 g.	
1	85	2–3	3	6·9	67,500	10
2	95	1–2	5	11·5	80,000	22
3	105	1–2	5	17·2	68,750	29
4	115	1–2	3	11·5	66,250	18
5	125	1–2	7	10·3	55,000	14
					Total recovery	93

Fraction 2 (run 20) had $E^{1\%}_{1\,cm.}$ 328 mμ = 1690 (in alcohol). The purest fraction which we have obtained by molecular distillation had $E^{1\%}_{1\,cm.}$ 328 mμ = 1950.

Crystallization of distilled vitamin A alcohol

A solution of vitamin A (20·4 g., Carr-Price value 81,000) in methyl alcohol (250 ml.) was cooled in solid carbon dioxide, inoculated with crystals obtained from a previous preparation, and allowed to warm up until only a small amount of solid remained undissolved. The bottle containing the solution was then covered with cotton wool and placed in a tin surrounded with solid carbon dioxide. After two days' storage at about −75°, the pale yellow crystals were collected on a sintered glass filter, cooled with solid carbon dioxide. The material was pressed on a porous plate and dried during 3 hr. in a high vacuum, at a temperature which was allowed to rise slowly from −8 to 4°. Yield 9·2 g.

This material when allowed to warm up slowly in an evacuated tube, melts at about 8° to a liquid which evolves vapour which may prove to be solvent of crystallization and simultaneously becomes more viscous. When completely freed from solvent in this manner, the product had Carr-Price value 92,000 and $E^{1\%}_{1\,cm.}$ 328 mμ = 1820 (in ethyl alcohol).

Vitamin A stearate

Vitamin A alcohol (17·4 g., Carr-Price value 80,000) in ether, was treated with ethereal solutions of recrystallized stearyl chloride (16·2 g.), and triethylamine (17·4 g.). The solutions of the latter reagents were added in parallel portions and the reaction was carried out at 0°, and in an atmosphere of nitrogen. The mixture stood overnight at room temperature and was filtered to remove triethylamine hydrochloride. The filtrate was washed with water, aqueous $NaHCO_3$ and water, and the ether was evaporated in nitrogen. In order to remove any unchanged vitamin A alcohol, the residue remaining after evaporation of the ether was dissolved in light petroleum (125 ml.), and the solution was allowed to percolate

through a column of activated alumina (150 g.). This material adsorbs the free vitamin A but not the esters. The column was rinsed with light petroleum and the filtrate and rinsings were evaporated under diminished pressure. The residue (17·3 g., Carr-Price value 48,000) consisted of a pale yellow oil which solidified to a wax at about 17°. This material was dissolved in ether and the solution was cooled in solid carbon dioxide; the pale yellow solid ester was collected and dried at a low temperature. The Carr-Price value of the product fell from 45,000 to 30,000 in 48 hr., and the experiment was therefore not pursued further.

Vitamin A diphenylacetate

Vitamin A (14 g., Carr-Price value 78,000) in pyridine (25 ml.) was treated with a solution of diphenylacetyl chloride (10 g.) in benzene (40 ml.). The mixture was warmed on the water bath for 60 min., then cooled and diluted with ethyl acetate. The solution was washed, dried and evaporated. The residue was dissolved in light petroleum and the solution was extracted ten times with an equal volume of 95 % methyl alcohol in order to remove unchanged vitamin A. The ester (4·9 g.) which remained after evaporation of the light petroleum had Carr-Price value 37,500, but could not be crystallized.

Vitamin A 4-nitrobenzoate

This ester, which was prepared in the usual manner by interaction between vitamin A and 4-nitrobenzoyl chloride in pyridine, formed a red oil which was soluble in ethyl acetate, acetone and ether, sparingly soluble in light petroleum, and insoluble in methyl alcohol. It was not obtained crystalline.

Vitamin A acetate

This compound, which was first described by Karrer [1931], was prepared by the interaction of distilled vitamin A (Carr-Price value 50,000) and redistilled acetyl chloride in pyridine. Since vitamin A and its acetate distil at approximately equal rates at the same temperature, the crude product was partitioned between methyl alcohol and light petroleum, in order to remove any vitamin that might have escaped esterification. For this, a solution of the acetate in light petroleum was extracted eight times with an equal volume of 95 % methyl alcohol. The acetate is somewhat soluble in methyl alcohol, and the process involved considerable loss. The acetate which was recovered from the petroleum layer had a Carr-Price value 34,000. It was diluted with residue oil and distilled. The most potent fraction distilled at 100° and consisted of a pale yellow, mobile oil with Carr-Price value 55,000 and $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ = 860.

Vitamin A anthraquinone-2-carboxylate

For the preparation of this ester, and of the 2-naphthoate, the method described by Hamano was used. The details are given in view of the difference in our starting material.

Distilled vitamin A alcohol (6·1 g., Carr-Price value 80,000) in pyridine (20 ml.) was treated with a solution of recrystallized anthraquinone-2-carboxyl chloride (4·3 g., M.P. 150°) in warm benzene (40 ml.). The mixture was heated on the water bath for 30 min. in an atmosphere of nitrogen, and the greater part of the solvents was then removed in a stream of this gas. The residue was dissolved in warm ethyl acetate (200 ml.). The solution was filtered and washed with water, dilute acid, water and aqueous $NaHCO_3$. After filtration to remove suspended sodium anthraquinone-2-carboxylate, and a final wash with water, the solution was dried (Na_2SO_4) and evaporated under diminished pressure. The

residue was dissolved in a small volume of acetone from which the ester soon began to separate in the form of small yellow plates. After two further crystallizations from acetone, the product formed macroscopic plates or prisms which melted at 126° (corr.) after turning red at about 123°. Hamano gives 124° as the melting point of the yellow modification of this ester. The Carr-Price value was 47,000 and the coefficient of absorption $E^{1\%}_{1\,cm.}$ 328 mμ = 938. These figures correspond to Carr-Price value 85,000 and $E^{1\%}_{1\,cm.}$ 328 mμ = 1510[1] for vitamin A alcohol. (Found C. 80·4, 80·7 %; H. 6·97, 6·81 %; $C_{35}H_{36}O_4$ requires C. 80·8 %, H. 6·92 %.)

The anthraquinone-2-carboxylate has also been obtained in the red form described by Hamano. This form was found to be less stable and more soluble at room temperature than the yellow allotrope. Conversion of the red into the yellow allotrope may be effected by solution in hot acetone, followed, after cooling, by inoculation of the solution with a crystal of the appropriate modification. After several recrystallizations from ethyl acetate-alcohol, the red modification formed long flat prisms having blunt ends, M.P. 118–120°. The Carr-Price value was 47,000 and the coefficient of absorption $E^{1\%}_{1\,cm.}$ 328 mμ = 1090. These figures correspond to Carr-Price value 85,000 and $E^{1\%}_{1\,cm.}$ 328 mμ = 1790[1] for vitamin A alcohol.

Vitamin A-2-naphthoate

Vitamin A alcohol (17·8 g., Carr-Price value 80,000) in pyridine (30 ml.) was treated in an atmosphere of nitrogen with 2-naphthoyl chloride (8·2 g.) in benzene (40 ml.). The mixture was heated during 30 min. on a water bath and the greater part of the solvents was then distilled off in a stream of nitrogen. The residue was taken up in ether. Further working as described for the anthraquinone-carboxylate gave a pale yellow syrup which was dissolved in acetone (*ca.* 30 ml.). The solution was cooled to about −75° as described by Hamano; the ester could not be induced to crystallize after several days at this temperature, and the solution was therefore treated with methyl alcohol (*ca.* 10 ml.). Crystallization started at once and was completed by cooling to −75°. The precipitate, which was partly crystalline and partly gummy, was collected and recrystallized from a small volume of acetone in which the ester is rather soluble. After four crystallizations from ethyl alcohol, the product weighed 950 mg. and melted at 78° (corr.). Hamano gives 76° as the melting point of vitamin A 2-naphthoate.

The ester crystallized from alcohol as large pale yellow plates, and from acetone as pale yellow elongated prisms. The specimen used for the biological test (Part II) had, immediately after its preparation: Carr-Price value 50,000 and $E^{1\%}_{1\,cm.}$ 328 mμ = 1180 corresponding to Carr-Price value 76,900 and $E^{1\%}_{1\,cm.}$ 328 mμ = 1790[2] for the vitamin A alcohol. (Found: C, 83·9, 83·7 %; H, 8·06, 8·18 %. $C_{31}H_{36}O_2$ requires C, 84·5 %; H, 8·18 %.)

Another specimen had $E^{1\%}_{1\,cm.}$ 328 mμ = 1190 and Carr-Price value 62,500. These values correspond to $E^{1\%}_{1\,cm.}$ 328 mμ = 1800[2] and Carr-Price value 96,000 for vitamin A.

The effect of storage on the properties of the crystalline esters

Samples of the batches of the anthraquinone-2-carboxylate and 2-naphthoate which were used for animal assays were stored in evacuated amber tubes. After about 3 months the esters were unaltered in appearance and had undergone no

[1] These values have been corrected for the absorption due to anthraquinone-2-carboxylic acid; ethyl anthraquinone-2-carboxylate was found to have ϵ_{328} = 5600.

[2] These values have been corrected for the ultraviolet absorption of 2-naphthoic acid for which we found ϵ_{328} = 820.

marked change in melting point. Further examination, however, disclosed that the ultraviolet absorption of both esters had decreased by about 20 % and that whereas the Carr-Price value of the naphthoate was practically unchanged, that of the anthraquinone carboxylate had decreased by about 15 %. This behaviour contrasted sharply with the apparent stability of the solutions of these esters in arachis oil, which were used for the biological tests. The effects of storage *in vacuo* are summarized in Table II. Exposure of the crystals to the air brought about rapid decomposition.

Table II

Ester ...	Anthraquinone-2-carboxylate		2-Naphthoate	
Date ...	5. iii. 38	13. vi. 38	17. iii. 38	13. iii. 38
$E^{1\%}_{1\,cm.}$ 328 mμ	938	750	1180	980
Carr-Price value	47,000	40,000	50,000	52,000
M.P. (corr.)	126° after turning red at 123°	127° after turning red at 121°	78°	77–79°

PART II

Biological tests were carried out on the yellow modification of vitamin A anthraquinone-2-carboxylate and on vitamin A 2-naphthoate. Both esters were dissolved in arachis oil containing 0·01 % quinol, the anthraquinone-2-carboxylate at a concentration of 0·15 % (solution P. 87) and the 2-naphthoate at 0·2 % (solution P. 90). The solutions were kept under nitrogen in the refrigerator, dilutions being prepared at regular intervals for administration to the animals: the diluted solutions were also kept under the same conditions except when required for feeding. The coefficients of absorption and the Carr-Price blue values of the two solutions (P. 87 and P. 90) were determined at intervals during the course of the experiments (Table III): the results show that no deterioration occurred during this period. (Later determinations of the blue values have shown that the solutions have remained stable for 7 months.)

The biological tests were carried out by us independently, employing the technique customary in each laboratory.

Table III

P. 87			P. 90		
Date	$E^{1\%}_{1\,cm.}$ 328 mμ	Carr-Price blue value	Date	$E^{1\%}_{1\,cm.}$ 328 mμ	Carr-Price blue value
16. iii. 38	—	64	22. iii. 38	2·14	100
17. iii. 38	1·42	64	28. iii. 38	2·15	100
21. iii. 38	1·43	64	11. iv. 38	2·14	96
11. iv. 38	1·51	60	26. iv. 38	2·10	96
26. iv. 38	1·55	60	14. vi. 38	2·03	100
14. vi. 38	1·55	60			

I. *Determination of biological potency by S. W. F. U.*

For the feeding experiments the solutions of the esters were further diluted with arachis oil containing quinol. P. 87 was diluted 1 : 6 and 1 : 12 and P. 90 1 : 10 and 1 : 20, that is, in both cases to make solutions with Carr-Price blue values of 10 and 5 respectively. Solutions containing 200 and 100 units per g. were similarly prepared from the solution of the international standard β-carotene. The dilutions were checked by determination of the blue value.

The biological tests were carried out on young rats which were given our vitamin A-free diet [Culhane, 1933] when they had reached a weight of about 40 g. each. When the weights became stationary the animals were divided into groups, those from different litters being distributed as evenly as possible amongst the different groups. All the groups received the dose of 0·02 g. of oil twice a week given by calibrated pipette directly into the animals' mouths. Three groups of each sex were given doses of the two esters and of the standard respectively. Another three groups of each sex were given a dose of ester or standard once a week only, alternately with a dose of arachis oil. The seventh group (negative controls) were given only doses of arachis oil twice a week.

Two experiments were carried out; in the first 78 animals were employed—51 bucks and 27 does; of these 8 bucks and 3 does were kept as negative controls. The doses were 0·02 g. of the solutions of the esters diluted 1 : 6 (P. 87) and 1 : 10 (P. 90) and four units of standard, given once and twice weekly. In the second experiment 57 animals were used—26 bucks and 36 does; 3 bucks and 4 does were kept as negative controls. The doses of the esters were 0·02 g. of the dilutions 1 : 12 (P. 87) and 1 : 20 (P. 90) with two units of standard, given once and twice weekly.

The average growth rate of the different groups in g. per week was calculated after the supplements had been given for three weeks. The results are given in Table IV.

Table IV

Exp. no.	Weekly dose	Dilution employed	♂ No. of rats	♂ Growth in g. per week	♀ No. of rats	♀ Growth in g. per week
		Vitamin A anthraquinone-2-carboxylate: P. 87				
1	0·04 g.	1 : 6	4	9·8	5	7·5
	0·02 g.	1 : 6	4	7·0	6	5·0
2	0·04 g.	1 : 12	2	5·5	4	5·4
	0·02 g.	1 : 12	3	4·4	5	1·4
		Vitamin A 2-naphthoate: P. 90				
1	0·04 g.	1 : 10	4	10·9	6	7·9
	0·02 g.	1 : 10	7	9·1	4	5·6
2	0·04 g.	1 : 20	3	4·3	4	5·3
	0·02 g.	1 : 20	4	2·3	4	1·8
		International standard				
1	8 units		8	8·8	7	6·0
	4 „		6	7·6	6	3·2
2	4 „		6	1·4	5	1·7
	2 „		3	−0·7	7	−1·5

Since standard dose-response curves for bucks and does are not in use in this laboratory, curves relating the increase in weight to the logarithm of the dose were first calculated for each solution P. 87 and P. 90 and for the international standard. The average slope of the three curves was next obtained, making allowance for the number of animals used, and a curve with this slope adjusted (mathematically) to the midpoint of each of the individual curves, so that the ratios of the doses of P. 87 and P. 90 to the dose of standard could be found. From these figures the potency of each solution was calculated, the results given by the bucks and does being averaged, with weighting for the number of animals used. The mean potencies are given in Table V.

Table V. *Potency of solutions P. 87 and P. 90 in units per g.*

Exp. no.	P. 87	P. 90
1	1580	3830
2	4250	4880
Mean	2740	4280
Standard error	+35 % −26 %	+36 % −27 %

In the first experiment the average growth rate was also determined for a period of 5 weeks: the potencies of the solutions calculated from these figures are not significantly different from those given in Table V.

The potencies of the esters and of vitamin A alcohol were calculated from the mean potencies of the solutions P. 87 and P. 90. The potency of the anthraquinone-2-carboxylate is 1,827,000 units per g. and of the 2-naphthoate 2,140,000 units per g. The figures for the potency of vitamin A alcohol are, respectively, 3,320,000 and 3,293,000 units per g. Since β-carotene contains, by definition, 1,667,000 units of vitamin A activity per g., vitamin A is, weight for weight, twice as potent as β-carotene.

II. *Determination of biological potency by K. H. C.*

The vitamin A potency of the two preparations was determined by the increase in weight brought about by giving doses to rats which had been fed on a vitamin A-free diet until they had become steady in weight. The details of the method have been fully described elsewhere [Coward, 1938].

The amount of vitamin A in P. 87 was theoretically 0·0825 % (mol. wt. vitamin A anthraquinone-2-carboxylate 520: mol. wt. vitamin A alcohol 286). The dose of solution tested was 0·5 mg. (diluted with arachis oil 1 : 20) per day in comparison with one unit of the international standard in 10 mg. arachis oil.

The amount of vitamin A in P. 90 was theoretically 0·13 % (mol. wt. vitamin A 2-naphthoate 440). The dose of solution tested was 0·33 mg. (diluted with arachis oil 1 : 30) per day in comparison with 0·5 mg. of the first solution and one unit of the international standard in 10 mg. arachis oil.

The results were calculated in the way in general use in the laboratories of the Pharmaceutical Society. They are summarized in Table VI.

Table VI

		No. of rats		Increase in wt.		Corresponding abscissae on curves of response		Weighted mean of abscissae
	Dose	♂	♀	♂ g.	♀ g.	♂	♀	
P. 87	0·5 mg.	5	9	13·0	15·3	1·72	2·48	2·21
P. 90	0·33 mg.	5	7	26·6	13·4	4·94	1·94	3·19
Vit. A	1 I.U.	4	10	22·5	10·0	3·60	1·25	1·92

P. 87. $$\frac{\text{The vitamin A content of 0·5 mg. P. 87}}{\text{1 I.U. of vitamin A}} = \frac{2·21}{1·92} = 1·15.$$

Therefore 0·5 mg. P. 87 contains 1·15 units vitamin A and the solution contains 2300 I.U. of vitamin A per g.

P. 90. $$\frac{\text{The vitamin A content of 0·33 mg. P. 90}}{\text{1 I.U. of vitamin A}} = \frac{3·19}{1·92} = 1·66.$$

Therefore, 0·33 mg. P. 90 contains 1·66 units vitamin A and the solution contains 5000 I.U. of vitamin A per g.

The potency of vitamin A according to the first test:

$$\text{Dose of vitamin A given was } 0{\cdot}0005 \times \frac{0{\cdot}0825}{100} \text{ g.} = 0{\cdot}0000004125 \text{ g.}$$
$$= 0{\cdot}4125\,\mu\text{g.}$$

$$\frac{0{\cdot}4125\,\mu\text{g. vitamin A}}{0{\cdot}6\,\mu\text{g. } \beta\text{-carotene}} = \frac{2{\cdot}21}{1{\cdot}92}.$$

Therefore vitamin A is 1·67 times as potent as β-carotene.

The potency of vitamin A according to the second test:

$$\text{Dose of vitamin A given was } 0{\cdot}00033 \times \frac{0{\cdot}13}{100} \text{ g.} = 0{\cdot}000000429 \text{ g.}$$
$$= 0{\cdot}429\,\mu\text{g.}$$

Therefore
$$\frac{0{\cdot}429\,\mu\text{g. vitamin A}}{0{\cdot}6\,\mu\text{g. } \beta\text{-carotene}} = \frac{3{\cdot}19}{1{\cdot}92}.$$

Therefore vitamin A is 2·32 times as potent as β-carotene.

The average of these two figures gives the potency of vitamin A as twice that of β-carotene, weight for weight.

Discussion

We consider that our results, which were obtained independently in different laboratories, show reasonably good agreement: they are summarized in Table VII.

Table VII

	P. 87 Vitamin A anthraquinone 2-carboxylate	P. 90 Vitamin A 2-naphthoate
Concentration of solution in arachis oil	0·15 %	0·2 %
$E^{1\%}_{1\text{ cm.}}$ 328 mμ	1·49	2·11
Carr-Price blue value	60	100
Potency: Units per g. S. W. F. U. 1	1580	3830
S. W. F. U. 2	4250	4880
K. H. C.	2300	5000
Mean	2625	4450
"Conversion factor" (corr.)	1920	2150

The potencies of the esters and of vitamin A alcohol calculated from these figures are:

	Units per g.		Units per g.
Vitamin A anthraquinone-2-carboxylate	1,750,000	Vitamin A alcohol	3,181,000
Vitamin A 2-naphthoate	2,225,000	Vitamin A alcohol	3,424,000

In the case of each ester the calculated potency of the vitamin A alcohol is approximately twice that of β-carotene.

For some years it has been assumed that the molecule of β-carotene breaks up in the animal body and by the addition of an OH group and an H atom to each of the broken ends, two new molecules are formed, each of which is a molecule of vitamin A. Thus 536 g. of β-carotene would give 572 g. of vitamin A and vitamin A and β-carotene would have very nearly equal activities weight for weight. But the two samples of vitamin A examined biologically have been shown to have 1·91 and 2·05 times respectively the vitamin A potency of β-carotene. (The difference between these figures is not significant.) This is in agreement with the results of Holmes & Corbet [1937] who found the potency of their vitamin A to be 3,000,000 I.U. per g. These results indicate either that (*a*) β-carotene is not converted quantitatively into vitamin A in the animal body or that (*b*) the molecule

of β-carotene gives rise to only one molecule of vitamin A, the other part of the molecule having no activity either when it was part of the carotene molecule or when it was separated from it. The latter possibility seems to be the more probable one since otherwise it is unlikely that the several tests should all have indicated that vitamin A has double the potency of β-carotene, weight for weight. Also it might be expected that, if β-carotene is not converted quantitatively into vitamin A in the body, the variation in different rats' powers of conversion would result in a greater variation in response to a given dose of carotene than that in response to a dose of any other form of vitamin A. Coward [1936] has shown that the standard deviation of the response of rats which had been given vitamin A in the form of carotene in vegetables was not greater than, and was apparently a little less than, that of rats given true vitamin A in cod liver oil, butters and vitaminized margarine (Table VIII).

Table VIII

	No. of rats from which the calculation was made		Standard deviation	
	♂	♀	♂	♀
1. Tests on a cod liver oil, Z	510	645	11·58	9·51
2. Tests on (*a*) butters and	257	315	12·75	9·72
(*b*) margarines	281	371	11·45	10·03
1 and 2 together	1048	1331	11·85	9·71
*Tests on β-carotene, the international standard	207	243	8·95	7·84

* Not before published.

A calculation has since been made of the standard deviation of the response of rats to doses of the international standard (i.e. β-carotene in oil) (Table VIII). It was found to be less than that of rats given doses of vitamin A in any other form and the difference was almost certainly significant.

$\frac{\sigma_1 - \sigma_2}{\sqrt{(\epsilon_1^2 + \epsilon_2^2)}}$, in which σ is the standard deviation of a single observation of increase in weight, and $\epsilon = \frac{\sigma}{\sqrt{\{2(n-m)\}}}$, n being the number of rats and m the number of groups of rats, was 5·06 for the male rats and 4·23 for the female rats. Thus the variation in response to doses of β-carotene in oil is less than the variation in response to doses of true vitamin A in oil, and therefore the β-carotene is probably not incompletely converted into vitamin A in the animal body.

It must be concluded, therefore, that the molecule of β-carotene has only one active group and that, on breaking down in the animal body, only one molecule of vitamin A is formed from one molecule of β-carotene, the other part of the carotene molecule presumably being converted into some substance which is without any vitamin A activity.

The fact that the calculated potency of vitamin A is the same in the case of each ester shows that the vitamin in each is assimilated by the rat to the same extent: it is of interest that esters which are not natural to the body can be apparently completely utilized. Our results taken in conjunction with those of Holmes & Corbet afford strong evidence that the rat utilizes indifferently and probably completely vitamin A and its natural esters. Utilization of the synthetic esters presumably depends upon their hydrolysis in the body: it will be of interest if a non-hydrolysable and, therefore, inactive ester is prepared in the future.

It has been stated [Wilkinson, 1938] that the curve of response for vitamin A is different from that for carotene. This can be tested for these experiments by comparing the increases in weight with the pairs of doses of esters and international standard in Table IV, since the slope of the curve for each pair of doses is formed by dividing the difference between the increases in weight by the difference between the logarithms of the doses given, which in these experiments is always 0·301. For the male rats, the differences between the increases in weight from pairs of doses of the esters were 2·8, 1·1, 1·8 and 2·0 and for the female rats, 2·5, 4·0, 2·3, 3·5; the corresponding figures from pairs of doses of the standard were for the male rats 1·2 and 2·1, and for the female rats 2·8 and 3·2. It is obvious, without further calculation, that the slopes of the curves of response for vitamin A are, under the conditions of this experiment at least, not different from those for β-carotene. Since pairs of doses were not given in the experiment recorded in Table VI, it is not possible to test these figures in the same way.

We have also calculated from our figures the value of the "conversion factor" for converting the value of the extinction coefficient of vitamin A into biological units per g. For this purpose it is necessary to correct the observed $E^{1\%}_{1\,\mathrm{cm.}}$ 328 mμ values (Table III) for the absorption due to the acid fraction of the ester molecules. The corrections are for P. 87, 0·12, and for P. 90, 0·04, and the observed values must be reduced by these amounts. The value of the conversion factor for the vitamin A anthraquinone-2-carboxylate solution is then 1920 and for the solution of the vitamin A 2-naphthoate, 2150: the difference between these figures is not significant. The factor accepted for the B.P. addendum 1936 is 1600, but our results are within the upper range of the figures from which the average of 1600 was obtained. They suggest, however, that the true value for the "conversion factor" for pure vitamin A is about 2000. Two explanations may be offered for the variations from this figure which have frequently been observed; either the solution examined has contained material which absorbs at wave lengths in the region of 328 mμ, but is not vitamin A or vitamin A in the form in which it is usually found, or the absorption of the vitamin from the intestine, or its utilization in the body of the rat varies according to its source, or according to the diet on which the animals are maintained. Our own results incline us to favour the former explanation although they do not exclude the latter.

Summary

Part I

1. Vitamin A has been obtained in a high state of purity by distillation in the Hickman cyclic still.

2. The distilled vitamin has been crystallized directly from methyl alcohol at a low temperature.

3. The stearate, 4-nitrobenzoate, diphenylacetate and acetate have been prepared from highly purified vitamin A, but have not been obtained crystalline.

4. The crystalline anthraquinone-2-carboxylate and 2-naphthoate of vitamin A have been prepared from material purified by molecular distillation. These esters were found to resemble the anthraquinone-2-carboxylate and 2-naphthoate prepared by Hamano from undistilled concentrates.

Part II

1. The biological potencies of two esters of vitamin A have been determined in terms of the international standard β-carotene in simultaneous tests.

2. Vitamin A anthraquinone-2-carboxylate was found to contain 1,750,000 units per g. and vitamin A 2-naphthoate 2,225,000 units per g.

3. The potency of vitamin A alcohol is therefore 3,181,000 I.U. per g. according to the tests on the first ester and 3,424,000 I.U. per g. according to the tests on the second ester.

4. Since, by definition, β-carotene contains 1,670,000 I.U. of vitamin A activity per g., vitamin A itself is twice as active as β-carotene, weight for weight.

5. The factor for converting the coefficient of absorption at 328 mμ into biological units per g. ("conversion factor") was found to be about 2000.

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LXXIII. THE WATER-SOLUBLE B-VITAMINS

XIII. ALLOXAZINE-ADENINE-DINUCLEOTIDE, ADENYLIC ACID, NICOTINAMIDE AND PIMELIC ACID IN THE NUTRITION OF THE RAT

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Recent investigations carried out in this laboratory have shown that the vitamin B requirements of the rat are satisfied only when the animals receive aneurin, riboflavin and three additional heat-stable factors belonging to what is known as the vitamin B_2 complex [Edgar *et al.* 1938, 3]. One of these factors, named yeast eluate factor, is contained in eluates prepared from fuller's earth adsorbates of autoclaved aqueous yeast extracts and has been identified [El-Sadr *et al.* 1939] with Lepkovsky's factor 1 [Lepkovsky, 1938] and György's vitamin B_6 [György, 1938, 1; Keresztesy & Stevens, 1938; Kuhn & Wendt, 1938]. A second factor, which so far has not been isolated in a pure state, is present in fuller's earth filtrates of autoclaved aqueous yeast extracts and in certain preparations from liver, and has been named yeast or liver filtrate factor according to the source. The existence of a third factor is indicated by the fact that rats receiving crude extracts of yeast or liver, as source of the vitamin B complex, increased in body-weight more rapidly than did rats receiving adequate amounts of aneurin, riboflavin, yeast eluate factor and yeast filtrate factor. Concentrates of this third factor have now been prepared by a method which will be described in a later paper.

A description is here given of experiments in which we studied the possibility of replacing for the nutrition of the rat certain of the above-recognized members of the vitamin B_2 complex by substances which, in other types of investigations, have been found to be biologically important. We have examined (A) alloxazine-adenine-dinucleotide, (B) a combination of yeast adenylic acid and nicotinamide and (C) pimelic acid.

A. *Alloxazine-adenine-dinucleotide.* Warburg & Christian [1938, 1] have recently isolated from liver and yeast a riboflavin-containing dinucleotide (alloxazine-adenine-dinucleotide), which is the coenzyme of *d*-amino-acid dehydrogenase, of xanthine dehydrogenase and of other enzymes [Warburg & Christian, 1938, 2; Ball, 1938; Haas, 1938]. It was to be expected that this important coenzyme would replace riboflavin in the diet of the rat, and it seemed possible that it might also possess the vitamin activity of some other member of the vitamin B complex. We have been able to investigate this problem through the kindness of Prof. Warburg, who put an adequate amount of the coenzyme at our disposal.

B. *Combination of yeast adenylic acid and nicotinamide.* Nicotinic acid or nicotinamide has been shown to be an essential nutrient for man [Fouts *et al.* 1937; Smith *et al.* 1937; Spies *et al.* 1938], the dog [Elvehjem *et al.* 1937], the pig [Chick *et al.* 1938, 1] and the monkey [Harris, 1938]. Frost & Elvehjem [1937]

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reported that nicotinamide and adenylic acid from yeast or heart had growth-promoting properties when administered, either separately or simultaneously, to rats receiving a basal diet which contained 12 % of white corn. Later, however, Oleson *et al.* [1939] found no growth-stimulating action of nicotinic acid when given to rats on an entirely "synthetic" diet containing riboflavin. Euler & Malmberg [1936] stated that adenylic acid had no effect on the growth-rate of rats receiving a diet containing riboflavin, but that nicotinamide prolonged the life of rats receiving riboflavin and a yeast fuller's earth filtrate. More recently cozymase was found to have a growth-stimulating effect when administered to rats receiving riboflavin and a fuller's earth filtrate of an autoclaved acidulated yeast extract [Euler *et al.* 1938].

In this laboratory we were unable to demonstrate that nicotinamide, nicotinic acid or codehydrogenase II were dietary essentials for the rat [Macrae & Edgar, 1937]. Cook *et al.* [1937] also failed to find that nicotinic acid alone or in combination with adenylic acid had any vitamin-like action in the rat. Chick *et al.* [1938, 2] noted no increased growth when rats fed on a maize diet received nicotinamide, and Chick (unpublished experiments) was also unable to demonstrate that nicotinamide had a growth-stimulating effect when fed to rats receiving a purified synthetic basal diet containing sucrose as source of carbohydrate. Helmer & Fouts [1938], on the other hand, reported that nicotinic acid did increase the growth-rate of rats receiving a maize diet containing riboflavin and liver filtrate fraction; György [1938, 2] has attributed an anaemia of rats, termed panmyelophthisis, to deficiency of nicotinic acid in the diet. Dann & Subbarow [1938] stated that nicotinic acid given to rats receiving a diet containing riboflavin had no growth-promoting action and did not prevent rat dermatitis, i.e. could not replace vitamin B_6.

In the present paper we report an extension of our former experiments [Macrae & Edgar, 1937] by investigating a combination of yeast adenylic acid and nicotinamide for vitamin activity.

C. *Pimelic acid.* Since the chemical behaviour of yeast filtrate factor proved it to be acidic in character [Edgar & Macrae, 1937, 2], it was thought possible that it might be identical with pimelic acid, a growth factor for *C. diphtheriae* [Mueller, 1937]. We have therefore tested pimelic acid for filtrate factor activity.

Methods and results

Growth of young rats was the criterion of vitamin activity employed; the basal diet and general experimental procedure have been described in previous papers from this laboratory [Chick *et al.* 1935; Edgar *et al.* 1937; 1938, 1]. All animals received 10 μg. aneurin and 0·08 ml. cod liver oil daily from the time of weaning until a body-weight of 100 g. was attained; the daily supplements were then increased to 15 μg. and 0·1 ml. respectively.

Synthetic aneurin and riboflavin were used throughout and the purified preparations of yeast and liver eluate factor and of yeast filtrate factor were those described in earlier papers [Edgar *et al.* 1937; Edgar & Macrae, 1937, 1; Edgar *et al.* 1938, 1, 2].

A. *Investigation of the vitamin B_2 activity of alloxazine-adenine-dinucleotide*

The sample of alloxazine-adenine-dinucleotide which we received from Prof. Warburg was a preparation of the monobarium salt obtained from yeast; it contained about 5 % of impurities. The material was fed to rats as the sodium salt, prepared by the addition of Na_2SO_4 to a solution of the barium salt.

Four separate experiments were carried out. Groups of rats received one of the following combinations of vitamin B_2 factors: (*a*) yeast eluate fraction and yeast filtrate fraction, (*b*) riboflavin and yeast eluate fraction, (*c*) riboflavin and yeast filtrate fraction, (*d*) riboflavin, yeast eluate and yeast filtrate fractions. The effect of the addition to the diets of alloxazine-adenine-dinucleotide on the growth-rate of the rats in these groups was observed.

(*a*) *Addition of alloxazine-adenine-dinucleotide to the diet containing yeast eluate factor and yeast filtrate factor (Table I A).*

These experiments were carried out to determine how efficiently the coenzyme replaced riboflavin in the diet of the rat.

Previous experiments had shown that rats receiving diets containing yeast filtrate and yeast eluate fractions, but no riboflavin, failed to grow, but gained rapidly in weight when riboflavin was subsequently added to the diet [cf. Edgar & Macrae, 1937, 1 (Fig. 2)]. The following experiment carried out in a similar manner with alloxazine-adenine-dinucleotide instead of riboflavin, indicated that this coenzyme could replace riboflavin in the diet of the rat. Two rats which received only yeast eluate and filtrate fractions as sources of the vitamin B_2 complex increased in weight at an average rate of 2·1 g. weekly for 3 weeks; after receiving an additional daily supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20 μg. riboflavin, the average weekly gain in weight during two subsequent weeks was 15·0 g.

A quantitative comparison of the vitamin activities of riboflavin and the coenzyme was, however, thought desirable.

The curve of response of body-weight increase to riboflavin dosage in animals receiving yeast eluate and yeast filtrate fractions as sources of the supplementary factors of the vitamin B_2 complex is a logarithmic one, similar to that obtained when yeast filtrate fraction is the only supplement given [Edgar *et al.* 1937]. We found optimum growth only when 40 μg. riboflavin daily were administered to each rat; the growth-rate, however, was most sensitive to small changes in the riboflavin content of the diet when the animals received only about one-quarter of the optimal amount.

Accordingly, the relative vitamin potencies of riboflavin and alloxazine-adenine-dinucleotide were determined by comparing the increase in body-weight of rats receiving only 6 or 12 μg. of riboflavin with that of animals receiving amounts of coenzyme equivalent to 6 or 12 μg. of riboflavin.

Animals from two litters of eight rats each received at weaning the basal diet supplemented by aneurin and cod liver oil. For the first few days the animals increased slightly in body-weight but this increase ceased after 7–10 days. Four of the animals from one of the litters each now received daily doses of yeast filtrate fraction purified by amyl alcohol extraction (=2 g. dry yeast), yeast eluate fraction (=2 g. dry yeast) and an amount of alloxazine-adenine-dinucleotide equivalent to 6 μg. riboflavin. Their four litter-mates received the same doses of yeast filtrate and eluate fractions but, instead of the coenzyme, 6 μg. of riboflavin daily. The rats of the second litter were similarly treated, but received double the amounts of coenzyme and riboflavin. The growth-rate of all rats was observed for 4 weeks after the supplements were given.

All rats showed immediate growth responses when the supplements were added (Table I A). The four animals receiving 6 μg. of riboflavin daily maintained an average growth-rate of 8·3 g. weekly for the 4-week period, and those receiving an equivalent amount of coenzyme gained an average of 7·4 g. weekly. Similarly,

Table I. *Effect of alloxazine-adenine-dinucleotide on the growth-rate of rats receiving aneurin and various other factors of the vitamin B complex*

A

No. of rats	Daily supplements of the vitamin B complex			Av. weekly wt. increases of group during 4 weeks after giving supplements g.	Mean weekly wt. increase g.
4	Yeast eluate factor = 2 g. dry yeast	Yeast filtrate factor = 2 g. dry yeast	6 μg. riboflavin	11·2, 6, 8·2, 8	8·3
4	,,	,,	Alloxazine-adenine-dinucleotide = 6 μg. riboflavin	9·5, 4, 8·5, 7·8	7·4
4	,,	,,	12 μg. riboflavin	19, 14, 14·5, 12·5	15·0
4	,,	,,	Alloxazine-adenine-dinucleotide = 12 μg. riboflavin	18·5, 15·2, 12·2, 9·8	13·9

B

No. of rats	Daily supplements during preliminary period of 2 weeks	Av. weekly wt. increases during preliminary period g.	Mean weekly wt. increase g.	Additional supplement added daily (= S)	Av. weekly wt. increases during 2 weeks after giving S g.	Mean weekly wt. increase g.
4	50 μg. riboflavin + yeast eluate factor = 2 g. dry yeast	18·5, 9·8	—	Alloxazine-adenine-dinucleotide = 20 μg. riboflavin	9·0, 5·5	7·2
2	,,	21·5, 8·5	—	Alloxazine-adenine-dinucleotide = 40 μg. riboflavin	11·5, 2·5	7·0
4	,,	20·2, 10	—	None	9·5, 5	7·2
1	,,	20, 12	—	Yeast filtrate factor = 1 g. dry yeast	17, 18	17·5

C

3	50 μg. riboflavin + yeast filtrate factor = 1 g. dry yeast	16, 13·3	14·6	Alloxazine-adenine-dinucleotide = 24 μg. riboflavin	13, 13·7	13·3
2	,,	14·5, 12·5	13·5	Liver eluate factor = 12 g. fresh liver	23·5, 22·5	23

D

No. of rats	Daily supplements of the vitamin B_2 complex	Alloxazine-adenine-dinucleotide given daily	Av. weekly wt. increases of group during 4 weeks after giving supplement g.	Mean weekly wt. increase g.
4	50 μg. riboflavin + eluate factor = 2 g. dry yeast + yeast filtrate factor = 2 g. dry yeast	Equivalent of 24 μg. riboflavin	19·8, 20·5. 20·5, 21·2	20·5
3	,,	None	22, 19·3, 21, 22·7	21·2

the two groups of animals receiving daily 12 μg. of riboflavin or alloxazine-adenine-dinucleotide equivalent to that amount of riboflavin increased in body-weight at approximately the same rate, the average weekly weight increases being 15 g. and 13·9 g. respectively. It is therefore concluded that, when administered orally, alloxazine-adenine-dinucleotide replaces riboflavin in the diet of the rat, and that equimolecular amounts of the coenzyme and riboflavin have the same vitamin potency.

(*b*) *Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast eluate factor* (*Table I B*).

Eleven rats received for 1 week from weaning the basal diet supplemented by aneurin and cod liver oil, and during a further preliminary period of 2 weeks each received daily, in addition, 50 μg. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. During the subsequent test period of 2 weeks, four of the animals received the additional supplement of an amount of alloxazine-adenine-dinucleotide equivalent to 20 μg. riboflavin daily, two received an amount equivalent to 40 μg. riboflavin, one serving as a positive control received an amount of yeast filtrate factor not purified by amyl alcohol extraction equivalent to 1 g. dry yeast, while four rats served as negative controls and received no further supplement. In this experiment the addition of alloxazine-adenine-nucleotide to the diet had no effect in restoring growth in the six rats receiving it (Table I B), the weekly weight increases being no greater than those of the negative controls. In the positive control rat, however, the growth-rate was restored.

(*c*) *Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin and yeast filtrate factor* (*Table I C*).

Five rats were maintained for 1 week from weaning on the basal diet supplemented by aneurin and cod liver oil, then for the further preliminary period of 2 weeks each received additional daily supplements of 50μg. riboflavin and of an amount of yeast filtrate factor not purified by amyl alcohol extraction equivalent to 1 g. dry yeast. During the subsequent test period of 2 weeks, daily supplements of the coenzyme equivalent to 24 μg. riboflavin were given to three of the rats, and eluate fraction, equivalent to 12 g. fresh liver, to the other two animals which served as positive controls. The addition of the coenzyme caused no increase in the growth-rate, while the addition of the eluate fraction caused the usual marked increase (Table I C).

(*d*) *Addition of alloxazine-adenine-dinucleotide to the diet containing riboflavin yeast eluate factor and yeast filtrate factor* (*Table I D*).

Seven male litter-mate rats at weaning received the basal diet supplemented by aneurin and cod liver oil for 1 week. Four of the animals then each received daily 50 μg. riboflavin, yeast eluate fraction equivalent to 2 g. dry yeast, yeast filtrate fraction purified by amyl alcohol extraction equivalent to 2 g. dry yeast, and alloxazine-adenine-dinucleotide equivalent to 24 μg. riboflavin. Three animals which served as negative controls received all these fractions except the coenzyme. There was no significant difference in the rates of growth of the two groups (Table I D), the average weekly gains in weight during a 4-week period being 20·5 and 21·2 g. respectively.

The usual weekly weight increase of similar male rats receiving unfractionated extracts of yeast or liver as sources of the whole vitamin B_2 complex is more than 30 g. The amounts of riboflavin and yeast filtrate and eluate factors administered were more than enough to supply the animals' needs of those factors; this experiment proves, therefore, (*a*) that at least one factor of the vitamin B_2 complex,

in addition to those factors, is required by the rat and (*b*) that alloxazine-adenine-dinucleotide cannot replace this additional factor.

The above experiments indicate that the only vitamin activity possessed by alloxazine-adenine-dinucleotide is that which it has by virtue of the riboflavin it contains bound in its molecule, and that when it is administered orally its vitamin potency is identical with that of an equimolecular amount of riboflavin.

Warburg & Christian [1938, 2] suggest that alloxazine-adenine-dinucleotide is probably the form in which riboflavin functions in the animal body, but it is also possible that riboflavin phosphoric acid or free riboflavin or other unknown compounds of riboflavin may be concerned with processes in the living cell. These experiments show, however, that riboflavin and alloxazine-adenine-dinucleotide, when administered orally to the rat, are converted with equal efficiency into the compound or compounds which function in the animal's tissues.

B. *Investigation of the vitamin B_2 activity of a combination of yeast adenylic acid and nicotinamide*

The yeast adenylic acid was obtained from British Drug Houses, Ltd., and the nicotinamide from Messrs Hoffmann La Roche. These compounds were tested for vitamin activity by administration to rats receiving supplements of (*a*) riboflavin, (*b*) riboflavin and yeast eluate fraction and (*c*) riboflavin and yeast filtrate fraction.

(*a*) *Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin (Table II A).*

Six litter-mate rats received for 1 week from weaning the basal diet supplemented by aneurin and cod liver oil. Three animals which served as negative controls then each received a daily supplement of 50 μg. riboflavin only, while the remaining three animals were each given daily 50 μg. riboflavin, 2 mg. yeast adenylic acid and 2 mg. nicotinamide; the doses of the last two of these compounds after 1 week were changed to 1 mg. of each daily. The body-weights of the animals were observed for 6 weeks (Table II A). The average total weight increase during the 6 weeks subsequent to the animals receiving adenylic acid and nicotinamide was 25 g., while the corresponding average for the control rats was 33 g. This difference, however, cannot be regarded as significant and there was no apparent difference in the general condition of the rats of the two groups; all the animals were much undersized but were active. Two rats of the control group receiving only riboflavin developed the characteristic florid rat dermatitis [Copping, 1936] after 6 and 15 weeks respectively from the beginning of the experiment, while two of the animals receiving riboflavin, adenylic acid and nicotinamide developed the dermatitis after 8 and 15 weeks respectively. Addition of adenylic acid and nicotinamide therefore did not influence either the growth of the rats or the development of dermatitis.

(*b*) *Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast eluate factor (Table II B).*

Four litter-mate female rats received at weaning the basal diet supplemented by aneurin and cod liver oil and for a preliminary period of 2 weeks additional supplements were given of 50 μg. riboflavin and yeast eluate fraction equivalent to 2 g. dry yeast. Three of the rats then each received for a further period of 3 weeks 2 mg. yeast adenylic acid and 2 mg. nicotinamide daily, while one animal received an amount of yeast filtrate fraction not purified by amyl alcohol extraction equivalent to 1 g. dry yeast. This last animal showed the usual growth

Table II. *Effect of a combination of yeast adenylic acid and nicotinamide on the growth-rate of rats receiving daily 10–15 μg. aneurin, 50 μg. riboflavin, and various other factors of the vitamin B_2 complex*

A

No. of rats	Daily supplements	Av. weekly wt. increases of group during 6 weeks after dosing g.
3	2 mg. adenylic acid + 2 mg. nicotinamide*	10·3, 4·7, 3·7, 2·3, 2·3, 1·7
3	None	15, 6·7, 5·0, 4·0, 1·0, 1·3

B

No. of rats	Daily supplements given during preliminary period of 2 or 3 weeks	Av. weekly wt. increases during preliminary period g.	Mean weekly wt. increase g.	Additional daily supplement added at end of preliminary period (S_1)	Av. weekly increases during 2 or 3 weeks after giving S_1 g.	Mean weekly wt. increase g.	Further supplement added (S_2)	Av. wt. increase during week after giving S_2 g.
3	Yeast eluate factor = 2 g. dry yeast	14·0, 9·3	—	2 mg. adenylic acid + 2 mg. nicotinamide	3, 6·3, 4	4·4	Yeast filtrate factor = 1 g. dry yeast	16·7
1	,,	16·0, 7·0	—	Yeast filtrate factor = 1 g. dry yeast	13, 18, 16	15·7	—	—
				C. Exp. 1				
3	Yeast filtrate factor = 1 g. dry yeast	20·3, 21, 13·7	18·3	2 mg. adenylic acid + 2 mg. nicotinamide	15, 17, 13·7	15·2	Yeast eluate factor = 2 g. dry yeast	24·3
				C. Exp. 2				
4	Yeast filtrate factor = 1 g. dry yeast	19·2, 15·2	17·2	2 mg. adenylic acid + 2 mg. nicotinamide	14·2, 15·2, 11·8	13·7	—	—
2	,,	18·5, 16·0	17·2	None	16, 16·5, 17	16·5	—	—
2	,,	21·5, 15·0	18·2	Yeast eluate factor = 2 g. dry yeast	31·5, 28·5	30	—	—

* After 1 week supplements of adenylic acid and nicotinamide reduced to 1 mg. of each daily.

response, but no increased growth-rate followed the administration to the test animals of adenylic acid and nicotinamide (Table IIB). At the end of the 3-week period addition of yeast filtrate fraction to the diet of these animals caused a striking increase in growth-rate, indicating that these amounts of adenylic acid and nicotinamide, although unable to stimulate growth, did not inhibit the growth of the animals when the missing nutrient was supplied. The increase in growth-rate caused by addition of filtrate factor, however, was not as great as that which follows administration of unfractionated yeast extracts to such animals; therefore, these compounds cannot replace that factor of the vitamin B_2 complex required in addition to riboflavin, eluate factor and filtrate factor.

(c) *Addition of yeast adenylic acid and nicotinamide to the diet containing riboflavin and yeast filtrate fraction (Table IIC).*

Three rats which had received the basal diet with aneurin and cod liver oil for 1 week from weaning were each given daily supplements of 50 μg. riboflavin and an amount of yeast filtrate fraction not purified by amyl alcohol extraction equivalent to 1 g. dry yeast for a period of 3 weeks; 2 mg. yeast adenylic acid and 2 mg. nicotinamide were then given daily for a further period of 3 weeks, but there was no increase in the growth-rate; increased growth-rate, however, did occur when a daily ration of yeast eluate fraction equivalent to 2 g. dry yeast was added (Table IIC, Exp. 1).

In a second experiment eight litter-mate rats, treated as usual for the first week after weaning, each received daily 50 μg. riboflavin and an amount of yeast filtrate fraction equivalent to 1 g. dry yeast for a period of 2 weeks. Four of the animals then each received the additional daily supplements of 2 mg. adenylic acid and 2 mg. nicotinamide for 3 weeks, two negative control animals received no additional supplement during that period and two positive control animals received yeast eluate fraction equivalent to 2 g. dry yeast daily. The animals receiving the adenylic acid and nicotinamide increased in body-weight at approximately the same rate as the negative controls while the growth-rates of the two positive control rats increased from an average of 18·2 g. weekly to 30 g. weekly on addition of the eluate factor (Table IIC, Exp. 2).

The results of the above experiments afford no evidence that yeast adenylic acid and nicotinamide administered simultaneously have any growth-promoting activity for rats deprived of the yeast eluate factor (vitamin B_6) or yeast filtrate factor of the vitamin B_2 complex; these compounds do not prevent rat dermatitis. We obtained no evidence that alloxazine-adenine-dinucleotide has any nutritional activity other than that due to the riboflavin it contains. Since this coenzyme contains also adenylic acid in its molecule, these experiments also suggest that adenylic acid is not an essential nutrient for the rat; additional support for this view was provided by experiments previously published which showed that codehydrogenase II (adenine-nicotinamide-dinucleotide) had no demonstrable nutritive value for rats [Macrae & Edgar, 1937]. It is of course possible that if nicotinamide and yeast adenylic acid were required by the rat in small amounts these might be contained in our purified yeast fractions or in our basal diet in such amounts that the rats were independent of a further supply, but it is at least certain that these compounds do not replace our yeast filtrate or yeast eluate factors in the diet of the rat. Recent experiments not reported and those described above also indicate that nicotinamide and adenylic acid cannot replace the additional unidentified factor which, with riboflavin, eluate

factor and filtrate factor, satisfies the vitamin B_2 requirements of the rat. The question whether nicotinamide and adenylic acid are essential nutrients for the rat will be decided only when all other factors are available in a pure state.

We have made no attempt to repeat the experiments of Frost & Elvehjem [1937], who found that a combination of yeast or heart adenylic acid and nicotinamide had very marked growth-promoting properties for rats receiving a diet which differed from that used by us principally in that it contained 12 % of white corn. The possibility cannot be excluded that white corn may have some property which renders the rat capable of benefiting from the administration of adenylic acid and nicotinamide; it appears to us, however, that the introduction of such complicated materials as cereals into basal diets used in the study of the different members of the vitamin B complex renders interpretation of the results difficult.

C. *Investigation of pimelic acid for yeast filtrate factor activity*

Pimelic acid was administered in the form of its sodium salt, which we received from Dr G. H. Jeffrey. The test for filtrate factor activity was carried out as previously described [Edgar *et al.* 1938, 1]. Three rats received none of the components of the vitamin B complex except aneurin for the first week after weaning and then riboflavin and eluate factor were added for a period of 2 weeks during which their weekly weight increases averaged 16·6 and 10 g. respectively. For a further period of 2 weeks the animals then received the additional supplement of 2 mg. pimelic acid, and the average growth-rates continued to fall to 4 and 1 g. weekly. Two positive controls which received an additional supplement of yeast filtrate factor when the other animals received the pimelic acid, increased in body-weight by approximately 20 g. weekly for the period of 2 weeks during which they were observed. Pimelic acid therefore does not replace filtrate factor in the diet of the rat.

Summary

1. Alloxazine-adenine-dinucleotide could replace riboflavin in the diet of the rat, equimolecular amounts of these compounds having the same nutritive potency. It had no demonstrable nutritive value other than its activity as a substitute for riboflavin.

2. A combination of adenylic acid and nicotinamide had no growth-promoting properties for rats receiving diets deficient in the yeast eluate factor (vitamin B_6) or the yeast filtrate factor of the vitamin B_2 complex, and with rats deprived of eluate factor did not influence the development of dermatitis.

3. Pimelic acid did not replace yeast filtrate factor in the diet of the rat.

We wish to thank Dr H. Chick for her interest and advice, Prof. O. Warburg who kindly presented the alloxazine-adenine-dinucleotide, Prof. A. R. Todd for the aneurin, Dr G. H. Jeffrey for the sodium pimelate, Dr M. Guggenheim of Messrs Hoffmann La Roche for the riboflavin and nicotinamide, and Dr B. K. Blount of Messrs Glaxo Laboratories, Ltd., for the liver extract used in the preparation of the liver eluate fraction. The yeast used in these experiments, as in previous experiments from this laboratory, was kindly given by Messrs Watney, Coombe and Reid.

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LXXIV. THE WATER-SOLUBLE B-VITAMINS
XIV. NOTE ON THE YEAST ELUATE FACTOR OF THE VITAMIN B_2 COMPLEX

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(*Received 1 March 1939*)

Biological and chemical investigation of the yeast eluate factor [Edgar & Macrae, 1937] had indicated that this factor was probably identical with vitamin B_6 [György, 1935] and factor 1 [Lepkovsky *et al.* 1936], and very potent concentrates of yeast eluate factor had already been prepared when it was reported that factor 1 [Lepkovsky, 1938, 1] and vitamin B_6 [Keresztesy & Stevens, 1938, 1; György, 1938; Kuhn & Wendt, 1938, 1] had been obtained in a pure state.

Dr Lepkovsky very kindly presented us with a generous sample of his crystalline factor 1 and we found that this material completely replaced our yeast eluate factor in the diet of the rat. Since the biological identity of yeast eluate factor and factor 1 was thus established, we did not pursue further our independent experiments on the isolation of yeast eluate factor. However, by submitting our concentrates to the methods of fractionation found successful for factor 1 [Lepkovsky, 1938, 2] we obtained with some difficulty a small amount of a crystalline material which appeared to be identical with the hydrochloride prepared from factor 1. m.p. of our crystals, 201–203°; mixed m.p. with the hydrochloride of factor 1, 202–203°. Our crystals had the same crystalline form as that of the hydrochloride of factor 1 and also gave similar red colorations with $FeCl_3$ [cf. Kuhn & Wendt, 1938, 2; Keresztesy & Stevens, 1938, 2]. The identity of vitamin B_6, factor 1 and yeast eluate factor is therefore established.

The activity of factor 1 in our rat growth tests for eluate factor

Rats were prepared as previously described [Edgar *et al.* 1938, 1] and received yeast filtrate factor and riboflavin for a preliminary period of 2 weeks. Some of the animals then received an additional daily supplement of 5μg. factor 1, others 10μg. and others 20μg. In all cases increases in the growth-rate of the animals occurred (Table I). The extent by which the growth-rate of the animals was increased by administration of factor 1 was of the same order as that which has been repeatedly observed following administrations of our yeast eluate factor to rats receiving the same diet with the same supplements. Crystalline factor 1 also cured the florid type of rat dermatitis [Chick, unpublished experiments].

The growth-rate increase following administration of 20μg. daily of factor 1 (free base) was somewhat greater than that observed with 10μg. daily, which in turn was considerably greater than that observed with 5μg. daily. The optimal daily requirement of the rat for factor 1 in growth experiments appears therefore to lie between 10μg. and 20μg. daily and is probably nearer 10μg. Lepkovsky [1938, 1] observed optimal growth when he administered 10μg. of factor 1

[1] Mission Member of the Egyptian University.

Table I. *Growth-promoting action of factor 1 for rats receiving a "vitamin B-free" basal diet with supplements of 10–15 μg. aneurin, 50 μg. riboflavin and yeast filtrate fraction equivalent to 1–2 g. dry yeast*

No. of rats	Av. weekly wt. increase of the group in each of 2 preliminary weeks g.	Av. weekly increase during 2 weeks g.	Factor 1 μg. daily	Av. weekly wt. increase of the group in each of 2 weeks subsequent to dosing with factor 1 g.	Av. weekly increase during 2 weeks g.
4	17, 12·5	14·7	5	20, 19·8	19·9
5	19·6, 13·6	16·6	10	26·2, 22·8	24·5
4	16·5, 14·5	15·5	20	28, 24·8	26·4
6*	18, 12	15	Yeast eluate fraction = 2 g. dry yeast	24, 25	24·5

* Taken from a previous paper [Edgar *et al.* 1938, 2 (Table II)].

(free base) daily and Dimick & Schreffler [1939] found that 10 μg. daily produced nearly optimal growth; Kuhn & Wendt [1938, 3] stated that the rate of weight increase observed following administration of 2·5–10 μg. daily of vitamin B_6 hydrochloride increased with the dose of the vitamin given.

We thank Dr H. Chick for her advice and criticism and Dr S. Lepkovsky for his generous gift of a specimen of crystalline factor 1. We are grateful to Messrs Hoffmann La Roche for gifts of riboflavin.

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LXXV. DIAPHORASE (COENZYME FACTOR)

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(*Received 21 February 1939*)

DIAPHORASE (coenzyme factor), one of the enzymes capable of reoxidizing reduced cozymase, has been the subject of a number of papers coming from this Institute and from Cambridge, where it was discovered independently and approximately simultaneously [Adler *et al.* 1937; Dewan & Green, 1937]. It is found widely distributed in animal tissues [Green *et al.* 1937; Dewan, 1938; Euler & Hasse, 1938], in yeast [Euler & Günther, 1938; Green & Dewan, 1938] and in several species of bacteria [Green & Dewan, 1938]. The results reported in this paper include an extension of the knowledge concerning the distribution of the enzyme in plant tissues, a further confirmation of its specificity as an oxidizing agent for coenzyme I, and a preliminary investigation of its action in respiratory systems other than those containing cytochrome carriers.

EXPERIMENTAL

Preparation of the enzymes. As sources of the enzyme both plant and animal tissues were used. Four plant materials, pea and bean seedlings, the thin outer layer of common white potato and a mould culture predominantly *Rhizopus* (sp.) and one animal tissue, pig heart muscle, were treated in the following way:

The seedlings were grown in metal trays on moist blotting paper for about 12 days. The cotyledons and leaves were then removed and the stems and roots ground to a pulp in a mortar with washed sand. One volume of $M/2$ Na_2HPO_4 was then added and the pulp reground until a homogeneous suspension was obtained. After an equal volume of water had been added, the coarsest particles and the sand were separated by straining through cloth and the remainder of the debris removed by centrifuging at 2500 r.p.m. for 5 min. The cloudy supernatant was treated with $\frac{1}{2}$ vol. sat. ammonium sulphate and the precipitate so obtained separated by centrifuging. A volume of $M/6$ secondary phosphate equal to the volume before addition of ammonium sulphate was added to the precipitate and the treatment with ammonium sulphate repeated. The precipitate finally obtained was suspended in half the original volume of $M/6$ Na_2HPO_4 buffer (pH 8·5). This method of preparation is a slight modification of that used by Euler & Hellström [1938] for animal tissue.

By the same procedure suspensions were prepared from potato peelings and from a mould culture which had been grown for about 2 weeks on moist bread.

The method of Euler & Hellström was used for the preparation of diaphorase from pig heart muscle. After the muscle had been reduced to a pulp and the red blood cells removed with 2 % NaCl, the decolorized mass was ground with sand and extracted with $M/2$ Na_2HPO_4. After dilution and centrifuging had been carried out for the first time, two CO_2-acetone precipitations at 0° were made. The precipitate obtained was suspended in $M/6$ Na_2HPO_4, treated with saturated ammonium sulphate, with CO_2 and acetone and finally with sat. ammonium sulphate again. The final precipitate was suspended in $M/6$ Na_2HPO_4 buffer (pH 8·5).

[1] American Scandinavian Foundation Fellow.

Stability. The stability of diaphorase from peas and beans differs quite markedly from that of diaphorase from pig heart. Although the latter retained its activity over a period of 4 months, the loss of activity in plant preparations was so rapid that preparations only 2 days old were unfit for experimental use. Similar observations have been made by several investigators for other materials.

Relative concentrations of enzyme in different tissues. It is possible only roughly to determine the relative concentrations of enzyme in plant and in animal material, since the same method of preparation cannot be used advantageously for both materials. However, when the times required for active enzymes prepared as described above to decolorize the same quantity of methylene blue in a standard Thunberg test are compared with the amount of material originally used, it is found that diaphorase in peas is only 1/6 and that in beans only 1/10 as concentrated as that in pig heart muscle. The same standard methylene blue test used to assay suspensions prepared from mould and from potatoes indicated that diaphorase was absent or nearly so from these preparations. These data are summarized in Table I.

Table I. *Concentration of diaphorase in plant and in animal tissues*

A complete system contains 0·25 ml. dihydrocozymase (1·2 mg./ml.), 0·25 ml. methylene blue (1/5000), 0·25 ml. diaphorase preparation and *M*/6 Na_2HPO_4 buffer (*p*H 8·5); total volume 1·50 ml. All tubes were incubated at 30°.

The controls indicate the spontaneous breakdown of dihydrocozymase in the presence of methylene blue and the absence of H-donating systems in the enzyme preparations.

	Material	System	Decolorization time min.	Relative concentration
1	—	Without enzyme	40	—
2	Pig heart	Complete	1·5	100
3	Pig, control	Without coenzyme	>120	—
4	Peas	Complete	6	16
5	Peas, control	Without coenzyme	>120	—
6	Beans	Complete	9	10
7	Beans, control	Without coenzyme	>120	—
8	Potato	Complete	42	—
9	Potato, control	Without coenzyme	>120	—
10	Mould	Complete	>120	—
11	Mould, control	Without coenzyme	>120	—

Specificity. The Thunberg methylene blue technique and the spectrophotometric technique were employed to investigate the specificity of plant diaphorase as an oxidant of dihydrocoenzymes I and II. The data obtained by the Thunberg method are presented in Tables II*a* and II*b*; those obtained by the spectrophotometric technique [Euler & Hellström, 1938] are shown in Fig. 1.

Spectrophotometric experiments. Exp. 1. (See below, (*a*) and (*b*).) The cell contained originally 1·0 ml. phosphate buffer (*p*H 7·2), 0·05 ml. diaphorase from peas, 3·7 ml. water and 0·15 ml. dihydrocodehydrase I (0·6 mg./ml.). After 10 min. 0·1 ml. methylene blue (1/5000) was added. Exp. 2. (See below, (*c*), (*d*) and (*e*).) The cell contained originally 1·0 ml. phosphate buffer (*p*H 7·2), 0·05 ml. diaphorase from peas, 0·85 ml. water and 3·00 ml. dihydrocodehydrase II (prepared enzymically). After 10 min. 0·1 ml. methylene blue (1/5000) and after 30 min. 0·1 ml. flavin enzyme were added. In both experiments the decrease in extinction at 334 mμ was measured.

(*a*) Dihydrocodehydrase I, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

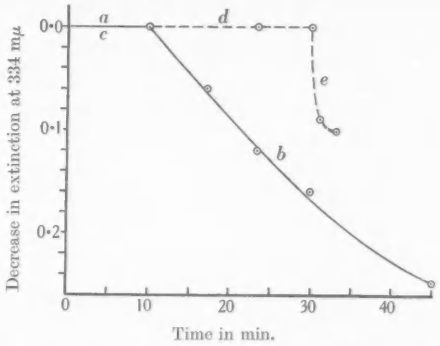

Fig. 1. Specificity of diaphorase for dihydrocodehydrase I.

Table II*a*. *Specificity test. Dihydrocodehydrase I (cozymase) and diaphorase*

A complete system contains 0·25 ml. dihydrocodehydrase I (1·2 mg./ml.); 0·25 ml. diaphorase from peas; 0·25 ml. methylene blue (1/5000) and phosphate buffer (*p*H 8·5) to make 1·50 ml. total volume.

	System	Decolorization time, min.
1	Complete	8
2	Without coenzyme	>80
3	Without diaphorase	37·5

Table II*b*. *Specificity test. Dihydrocodehydrase II*[1] *and diaphorase*

A complete system contains (*a*) 0·25 ml. Robison ester; (*b*) 0·25 ml. Robison ester dehydrogenase; (*c*) 0·25 ml. codehydrase II; (*d*) 0·25 ml. diaphorase from peas; (*e*) 0·50 ml. methylene blue (1/5000); (*f*) phosphate buffer (*p*H 8·5) to make a total volume of 1·75 ml.

In tube 6 diaphorase has been replaced by 0·25 ml. flavin enzyme.

	System	Decolorization time, min.
1	Complete	28·5
2	Without (*a*)	>120
3	Without (*b*)	>120
4	Without (*c*)	>120
5	Without (*d*)	28·5
6	Flavin enzyme	9·5
7	Without (*a*) and (*b*)	>120
8	Without (*a*), (*b*) and (*c*)	>120

(*b*) As (*a*) but with methylene blue added. Decrease in extinction = 0·24 unit in 35 min.

(*c*) Dihydrocodehydrase II, diaphorase and buffer. No reaction in 10 min. No autoxidizable flavoprotein or cytochrome present.

(*d*) As (*c*) but with methylene blue. No reaction in 20 min. No reaction between coenzyme and diaphorase.

(*e*) As (*d*) with flavin enzyme. Decrease of 0·1 unit in 3 min. Oxidizable coenzyme present.

The results of these experiments confirm the fact already reported by this Institute that diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).

[1] The dihydrocodehydrase II was formed *in situ* from codehydrase II and H made available from Robison ester by the action of Robison ester dehydrogenase.

The role of diaphorase in non-cytochrome systems

No statement has been made concerning the possible role of diaphorase in respiratory systems other than those involving cytochrome carriers. One system found in some plants both of higher and lower orders is the system in which quinones play a dominating part as H acceptors. It was felt that the best method of approach would be to study reactions involving dihydrocozymase and simple quinones, both in the presence and in the absence of diaphorase.

Procedure. Methods which employed the Thunberg technique and the Zeiss photometer and depended upon the disappearance of colour from solutions of quinones after the addition of dihydrocozymase were found to be inapplicable to this work. The method finally adopted depends upon the destruction of cozymase formed during the reaction between dihydrocozymase and quinone by heat in a strongly alkaline solution and subsequent measurement of residual dihydrocozymase by a fermentation technique [Myrbäck, 1933].

One set of solutions was prepared as follows: 2·0 ml. quinone (equivalent to 350 μg. coenzyme), 0·17 ml. diaphorase and 0·13 ml. dihydrocozymase (2·6 mg./ml.). A second set of solutions contained no diaphorase but an equal volume of $M/6$ Na_2HPO_4 buffer (pH 8·5). Controls containing neither dye nor diaphorase were also prepared. One tube of the first set and a corresponding tube of the second set were allowed to react for a definite period, after which the reaction was stopped by adding 0·50 ml. $N/10$ NaOH, to give a pH >10. The tubes were sealed and heated in boiling water for 10 min., then the solution was brought back to pH 8 by adding 0·50 ml. $N/10$ acetic acid. Pairs of tubes were treated in the same manner after the reactions had continued for varying times. Two controls were carried through the same procedure, the one heated after 15 min. and the other not heated at all. By this procedure any cozymase formed during the reaction was destroyed.

A fermentation mixture corresponding to each solution to be tested for residual dihydrocozymase contained the following components: 400 mg. apozymase, 100 mg. glucose, 0·45 ml. phosphate buffer (pH 6·3), 0·15 ml. hexosediphosphate, 1·05 ml. water and 0·35 ml. test solution. For best results an untreated test solution should contain between 50 and 100 μg. coenzyme per ml. Fermentations were carried out at 30° and the fermentation rate for each test solution determined from the volume of CO_2 evolved during the hour of most rapid fermentation. The fermentation rate is a measure of the amount of dihydrocozymase remaining in the original solution. The results of the reaction between dihydrocozymase and three quinones, o- and p-benzoquinone and β-naphthoquinone, both in the presence and in the absence of diaphorase, are shown in Fig. 2.

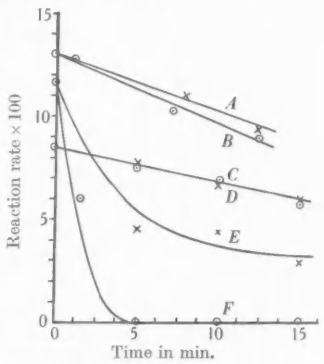

Fig. 2. Reduction of quinones by dihydrocozymase. Effect of diaphorase. Curve A, × × × o-benzoquinone without diaphorase. Curve B, ⊙ ⊙ ⊙ o-benzoquinone with diaphorase. Curve C, × × × p-benzoquinone without diaphorase. Curve D, ⊙ ⊙ ⊙ p-benzoquinone with diaphorase. Curve E, × × × β-naphthoquinone without diaphorase. Curve F, ⊙ ⊙ ⊙ β-naphthoquinone with diaphorase.

o-Benzoquinone was prepared according to a modification of methods reported by Kvalnes [1934] and by Willstätter & Pfannenstiel [1904] and

Willstätter & Müller [1908] starting with pyrocatechol, Ag_2O and fused Na_2SO_4 in absolute ether and light petroleum. β-Naphthoquinone was purified by recrystallization from alcohol. *p*-Benzoquinone was purified by recrystallization from alcohol and sublimation. The diaphorase used throughout the work was prepared from pig heart muscle. There was no loss of activity during the period of investigation.

The results indicate, in the cases of *o*- and *p*-benzoquinones, that diaphorase apparently plays no part in the transfer of H from dihydrocozymase to these simple quinones, but, in the case of β-naphthoquinone, that diaphorase accelerates H-transfer and that the reaction is extremely rapid during the first minutes. From these results no definite conclusion can be drawn concerning the part, if any, played by diaphorase in a quinone respiratory system. If, however, the result obtained with β-naphthoquinone can be discounted, the fact that two plant tissues (potato and mould), containing quinone respiratory systems, have little or no diaphorase parallels the observation that diaphorase did not accelerate the oxidizing action of the other quinones. A tentative conclusion, then, is that diaphorase is not necessary in respiratory systems involving quinones. This will be confirmed or denied by future investigation.

Summary

1. Diaphorase is present in the tissues of some higher plants and absent or nearly so in those of other higher plants and fungi.

2. Plant diaphorase is extremely unstable.

3. Plant diaphorase is a specific oxidant for dihydrocodehydrase I (cozymase).

4. The role of diaphorase as H transporter in respiratory systems other than those containing cytochrome carriers is still undetermined, although tentatively it is concluded that the enzyme is not necessary in quinone systems.

The author wishes to make known his appreciation of the counsel and laboratory aid given him by Prof. H. von Euler and his assistants, Drs F. Schlenk and E. Adler. The spectrophotometric measurements were kindly made by Mr G. Günther.

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LXXVI. EXPERIMENTS ON THE CHEMOTHERAPY OF CANCER

III. THE INDEPENDENCE OF TISSUE RESPIRATION AND GLYCOLYSIS, AND THE GROWTH RATE OF TUMOURS

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(*Received 28 February 1939*)

The main activity of tumours appears to be growth and hence it might be expected that growth rate and respiration or glycolysis would be proportional to each other under different conditions since the energy used for growth processes is probably obtained from carbohydrate metabolism. The respiration or anaerobic glycolysis of a stimulated uninjured muscle is almost proportional to the work performed [Meyerhof, 1930]. Stimulation of isolated submaxillary gland tissue with acetylcholine, which increases secretion, also increases respiration [Deutsch & Raper, 1938]. Murphy & Hawkins [1925], however, found the respiration and glycolysis of spontaneous mouse tumours to be independent of rate of growth.

The inhibition of tumour growth in rats by irradiation with X-rays is not accompanied by marked changes in glycolysis until a very considerable degeneration of tissue has occurred [Bancroft *et al.* 1935]. Some reduction of respiration may occur: in the experiments on rats given by Bancroft *et al.* the reduction is significant in about half the treated animals. In experiments of Crabtree [1932], in which rats bearing the Jensen rat sarcoma were treated with radium, an increase in the anaerobic and aerobic glycolysis of the tumour tissue was sometimes produced and the respiration was generally reduced, but growth of the tumours continued. The inhibition of growth of tumours by radiation does not appear to be necessarily dependent on inhibition of glycolysis or respiration.

In the case of muscular activity it has been shown that the decomposition of creatine phosphate is a more fundamental or essential change for activity than respiration and glycolysis. The inhibition of glycolysis with iodoacetic acid does not completely inhibit muscular activity. It now seems probable that analogous fundamental processes may govern tumour growth as creatine phosphate breakdown limits muscular action. In the present paper it is shown that the effect of some tumour-growth-inhibiting substances does not change the respiration and glycolysis of tumour tissue. In the search for compounds likely to be useful for the chemotherapy of cancer it may be considered that effective substances need not necessarily affect these processes although they are the largest changes which occur in tumour cells. It is proposed to determine which cell constituents or biochemical processes vary with growth rate or are affected by treatment with the known tumour inhibitors. It is hoped that such results may give a rational basis for further experiments in therapy.

Growth rates and metabolism of different tumour strains. The growth rate of different strains of transplanted tumours in mice varies from 0·01 to 1·35 mm. per day, measured as increase in average diameter [Boyland & McClean, 1935, see Table I]. Values for the respiration (Q_{O_2} from $-7{\cdot}5$ to -12) and glycolysis

[1] Finney-Howell Fellow.

Table I. *Growth rates and tissue metabolism of different mouse tumours*

Tumour	Mean growth rate (mm. increase in diam. per day)	Respiration $-Q_{O_2}$ Mean (min.–max.)	Glycolysis $Q_L^{N_2}$ Mean (min.–max.)
S 37	1·35	11·5 (8·5–13·1)	33·5 (23·3–39·2)
Crocker 180	0·88	8 (7·2– 9·4)	23 (20·5–26·2)
M 4	0·74	8·5 (7·6–10·9)	26 (22·1–34·0)
M.C.D.B.I.	0·70	10 (9·1–11·8)	28·5 (21·7–37·5)
Mal.	0·69	7·5 (7·2– 8·5)	25 (17·7–28·5)
C 63	0·66	12 (6·2–14·4)	25 (16·5–27·5)
L.M. 154	0·032	9·5 (8·0–10·2)	22 (20·2–25·6)
Melanoma (Passey)	0·010	8·5* (5·6–11·7)	16* (8·9–23·7)

* From Crabtree [1929].

($Q_L^{N_2}$ from 16 to 34), which were determined in the usual way in the Warburg apparatus at 37·5°, show much smaller variations which do not run parallel with growth rate. It should be remembered that the measurements of respiration and glycolysis of isolated tissues thus determined are measurements of ability to glycolyse and respire under artificial conditions and may be different from the rates of the tissues *in situ*. The rates of glycolysis observed probably represent the maximum values, but if either the ability to respire or glycolyse were the factors limiting growth they should vary with rate of growth of the tumour.

It would be of interest to determine whether glycolysis and respiration of different tumours growing in tissue culture correspond with the growth rate of the culture. This would allow determination of growth and metabolism under the same conditions, and then all determinations would be comparable.

Effect of treatment on tumour growth and metabolism. Two types of tumour were used. (*a*) Crocker sarcoma 180 grafted into mixed stock mice in the usual way. (*b*) Sarcoma M.C.D.B.I. originally obtained by subcutaneous injection of 1 mg. methylcholanthrene dissolved in 0·2 ml. olive oil into a male mouse of the pure Dilute Brown strain. The tumour has always been grafted into mice of the same strain, in which it grows readily and regularly: it is now in the eighth grafted generation. A tumour of this type, which has never grown outside the same pure line of mice, resembles spontaneous or induced tumours in that genetic differences between the tumour tissue and the host's tissues are probably unimportant. In the case of the Crocker sarcoma inhibition of growth on treatment may be due either to (1) increase in resistance of the host to a foreign tissue or (2) damage to the tumour tissue *per se*. In the case of the M.C.D.B.I. tumour the factor (1) should not operate. This latter tumour has some advantages over spontaneous tumours, viz. (*a*) it is more easily available in large numbers, (*b*) tumours in different mice grow at the same uniform rate so that some mice may be used as controls, (*c*) the chemical constituents and biochemical reactions are probably more constant.

Method. As soon after grafting as possible the size of tumours was measured with calipers every second day. Sodium sulphanilyl-sulphanilate and *pp'*-diaminodiphenylsulphoxide, the inhibiting action of which has been described [Boyland, 1938] and 4:4'-diamino-2:2'-dinitrodiphenylmethane and α-nitroso-β-naphthol, which have since been found to inhibit tumour growth, were administered by daily injection into the stomachs of mice, generally commencing on the day on which the tumours were grafted. The carcinogenic substances 1:2:5:6-dibenzanthracene and 1:2:5:6-dibenzfluorene, which Haddow *et al.* [1937] and Haddow [1938] have shown to inhibit tumour growth, were injected intraperitoneally as a single dose dissolved in oil about a fortnight after

grafting the tumour. Trypan Blue (as calcium salt) and Isamine Blue (as sodium salt), both of which stain tumour tissue, were injected intraperitoneally in four or five doses of 0·25 ml. of a 1 % aqueous solution, the first doses being given about a fortnight after grafting the tumours. After a suitable interval of treatment the mice were killed and the duplicate determinations of respiration and glycolysis of the tumour tissue were made in the Warburg apparatus. Occasionally the tumour growth was not inhibited; such tumours (except those in mice treated with Isamine Blue) were not used in the measurements.

Healthy tissue free from necrotic material was used for the determinations of metabolism. No obvious difference in the extent of necrosis in normal and inhibited tumours was observed, but closer investigation of this point will be made. Tumours inhibited by 1:2:5:6-dibenzanthracene or 1:2:5:6-dibenzfluorene often appeared to have a more fibrous structure than the controls and in these cases the fibrous material was necessarily included in the samples used for experiment.

Table II. *The effect of treatment on growth rates and tissue metabolism of mouse tumours*

Mouse treated with	Dose mg.	Mean inhibition of growth %	No. of mice	Respiration ($-Q_{O_2}$) mean ($\bar{x}$)	σ	$\frac{\bar{x}_1-\bar{x}_2}{\sqrt{(\epsilon_1^2+\epsilon_2^2)}}$	Glycolysis ($Q_L^{N_2}$) mean ($\bar{x}$)	σ	$\frac{\bar{x}_1-\bar{x}_2}{\sqrt{(\epsilon_1^2+\epsilon_2^2)}}$
				Sarcoma M.C.D.B.I.					
Controls		—	(9)	10·5	±1·7	—	29·6	±5·2	—
1:2:5:6-Dibenzanthracene	15	100	(6)	10·0	±1·2	<1	26·9	±2·2	<1
1:2:5:6-Dibenzfluorene	5	66	(5)	7·6	±2·4	1·4	26·6	±0·9	1·8
Sodium sulphanilyl-sulphanilate	50 daily	75	(6)	9·8	±0·4	1·1	29·0	±4·2	<1
pp'-Diaminodiphenyl-sulphoxide	8 daily	50	(5)	9·5	±1·2	1·3	23·0*	±2·5	3·2
4:4'-Diamino-2:2'-dinitro-diphenyl-methane	8 daily	30	(6)	10·3	±0·8	<1	28·4	±3·3	<1
α-Nitroso-β-naphthol	8 daily	33	(5)	9·4	±1·2	1·4	27·3	±6·0	<1
Trypan Blue	2·5 daily	83	(6)	6·3*	±2·7	3·5	22·6*	±4·7	2·7
Isamine Blue	2·5 daily	0	(4)	10·3	±0·4	<1	26·2	±2·0	1·6
				Crocker sarcoma 180					
Controls		—	(9)	8·1	±0·7	—	22·8	±2·1	—
1:2:5:6-Dibenzanthracene	15	66	(10)	7·8	±1·5	<1	22·5	±4·3	<1
1:2:5:6-Dibenzfluorene	5	60	(6)	7·5	±1·4	<1	25·4	±3·7	1·6
Sodium sulphanilyl-sulphanilate	50 daily	50	(5)	8·3	±0·7	<1	24·2	±1·3	1·4
pp'-Diaminodiphenyl-sulphoxide	8 daily	67	(7)	8·3	±1·5	<1	25·0	±2·5	1·8
4:4'-Diamino-2:2'-dinitro-diphenylmethane	8 daily	45	(6)	9·0	±1·1	1·8	24·2	±2·2	<1
α-Nitroso-β-naphthol	8 daily	50	(5)	7·6	±1·5	<1	19·1	±3·5	2·0
Trypan Blue	2·5 daily	63	(7)	4·7*	±1·6	5·3	13·2*	±6·5	3·8
Isamine Blue	2·5 daily	25	(5)	6·5	±1·9	1·6	18·8	±6·5	1·2

$\epsilon = \sqrt{\frac{\Sigma(\bar{x}-x)^2}{n(n-1)}}$ where $\bar{x}$ is the mean of the series of observations, x = any individual value and n = the number of observations. σ = standard deviation.

* These values are considered to be probably significantly different from the means for the corresponding control tumours—i.e. the values of $\frac{\bar{x}_1-\bar{x}_2}{\sqrt{\epsilon_1^2+\epsilon_2^2}} > 2$, where $\bar{x}_1$ = mean value for controls, $\bar{x}_2$ = mean value of the series under consideration.

Results

The results of this part of the experiment are given in Table II and show that with marked decrease in rates of growth, the metabolism is often not reduced. Significant reductions were found only (1) in the respiration and glycolysis of tumours in mice treated with Trypan Blue, and (2) in the glycolysis of M.C.D.B.I. from mice treated with *pp'*-diaminodiphenylsulphoxide.

Summary

1. The glycolysis and respiration of several strains of grafted tumours with growth rates varying from 0·01 to 1·35 mm. per day are not proportional to the rate of growth and vary within a narrow range.

2. The values for glycolysis and respiration of tissue from grafted tumours, the growth of which was inhibited by administration of 1:2:5:6-dibenzanthracene, 1:2:5:6-dibenzfluorene, sodium sulphanilylsulphanilate, α-nitroso-β-naphthol and 4:4'-diamino-2:2'-dinitrodiphenylmethane, were not significantly lower than values of untreated tumours.

3. The inhibition of growth produced by Trypan Blue was accompanied by decrease in respiration and glycolysis while that produced by *pp'*-diaminodiphenylsulphoxide was accompanied by reduction in glycolysis only.

4. Injection of Isamine Blue did not inhibit tumour growth nor reduce the metabolism.

We should like to thank Dr Smith of the Wellcome Chemical Works for samples of sodium sulphanilylsulphanilate and *pp'*-diaminodiphenylsulphoxide, and Prof. J. W. Cook for a specimen of 1:2:5:6-dibenzfluorene.

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LXXVII. SOME PROPERTIES OF EGG-WHITE LYSOZYME

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An enzyme capable of lysing certain bacteria, and particularly *M. lysodeikticus*, was discovered in nasal mucous by Fleming [1922] and called by him *lysozyme*. This, or a similar substance, is present in a number of animal tissues and fluids, the richest source being hen's egg-white.

An attempt to isolate lysozyme from egg-white was made by Wolff [1927] by removing inert protein with colloidal iron and precipitating the active material with acetone; the preparation obtained in this manner was said to contain no nitrogen or sulphur. Meyer *et al.* [1936] used an entirely different procedure. Acetone-dried egg white was extracted with 50% alcohol containing 10% acetic acid and the lysozyme precipitated from the concentrated solution with alcohol. It was purified by precipitation as the flavianate and decomposition of the latter with cold alcoholic ammonia. These investigators recognized the protein nature of the substance and considered that its lytic action involved the hydrolysis of a sugar linkage of certain mucoids present in the bacteria. The method of isolation was further improved by Roberts [1937]. Owing to the difficulty of decomposing the flavianate this part of the preparation was replaced by a fractional precipitation with acetone from a solution of the right acidity. The lysozyme was concentrated largely in the fourth and final fraction, which was said to behave in all respects like a homogeneous product, and had an activity, as determined by the method of Goldsworthy & Florey [1930], of about 2000 units per mg. This preparation was moderately soluble in water, showed most of the characteristic protein reactions, and its isoelectric point was thought to be near *p*H 7·0. Its dialysability through cellophane suggested that it was a protein of relatively low molecular weight.

The lysozyme used in the work described here was obtained as a white powder, dried with acetone and ether, by the method of Roberts. It had an activity of about 2000 units per mg. and contained 16·4% N and 3·2% S, calculated on a moisture- and ash-free basis. Ultra-centrifugal measurements by Mr J. St L. Philpot, using the absorption method, indicated that the material was homogeneous and was one of the group of proteins whose molecular weights are close to 18,000. Crystalline lysozyme was obtained from this material by Abraham & Robinson [1937] and appeared to have about the same activity as the amorphous substance, but unfortunately it has so far proved difficult to obtain crystals in sufficient quantity for chemical investigation.

In the meantime the properties of the amorphous substance, which was believed to possess a considerable degree of homogeneity, were further investigated. After an analysis had been made of certain of its constituent amino-acids it was decided to carry out an electrometric titration. Preliminary experiments in this connexion showed that the material was not completely homogeneous but that a certain fraction of it had a lower activity and much lower solubility than the rest.

Colorimetric estimation of certain amino-acids

Experimental

1·26 g. of the protein enzyme (prepared by the method of Roberts) were hydrolysed by boiling with 100 ml. of 20% HCl for 6 hr. and most of the acid removed by evaporating the solution several times to dryness. After removing the humin by the method of Keilin & Hartree [1937] the concentrated solution of amino-acids was diluted to 100 ml. and divided into two parts (solutions A and B).

Tyrosine was estimated in solution A by the method of Folin & Ciocalteu [1927] and cystine by the method of Folin & Marenzi [1929].

Solution B was evaporated *in vacuo* almost to dryness and the residue dissolved in 50 ml. of a solution containing 10 ml. of 33% HCl and heated on the water bath. 50 ml. of hot 15% phosphotungstic acid solution were then added and the mixture digested on the water bath for half an hour. After standing for 2 days the precipitate of diamino-acids was filtered and washed with 100 ml. of a 7·5% phosphotungstic acid solution containing 10 ml. of 33% hydrochloric acid. The precipitate and filter paper were then transferred to a beaker, dissolved in just sufficient 3% NaOH, and the solution filtered into a volumetric flask and diluted to 500 ml. (solution C).

Arginine was estimated in solution C by the method of Jean [1934] and histidine by the method of Jorpes [1932]. The values obtained were corrected for the solubilities of the phosphotungstates, using the figures given by Van Slyke [1911].

The total N in solution C was determined by the micro-Kjeldahl method and the lysine present estimated by subtraction from the total N of the arginine-, histidine- and cystine-N. To obtain a value for the latter a cystine determination was made on solution C.

Results

The values obtained in this manner were: arginine 11·6%, cystine 7·0%, lysine 5·8%, tyrosine 4·4%, histidine 2·6%.

The value for cystine may be high as the starting material gave a weak positive test for sulphydryl groups [Mirsky & Anson, 1936].

Calculated for a molecular weight of 18,000 the number of basic groups per molecule, due to arginine, lysine and histidine, is about 22.

Isolation of arginine

1·2 g. of protein were hydrolysed with HCl and the diamino-acids separated by precipitation as their phosphotungstates in the usual manner. 150 ml. of 1% HCl were added to the precipitate and the phosphotungstic acid extracted with 1 : 1 ether-amyl alcohol. The aqueous solution was evaporated to dryness, the residue dissolved in 15 ml. of water, heated to 90°, and 15 ml. of a solution containing 1 g. of flavianic acid added. After standing for 24 hr. in the ice chest the precipitate was centrifuged and crystallized from 110 ml. of boiling water containing a trace of flavianic acid (220 mg.). It was recrystallized from 60 ml. of hot water and dried at 100° over P_2O_5. (Found: N, 17·47%. $(C_{10}H_6N_2SO_8)$ $(C_6H_{14}O_2N_4)$ requires N 17·2%.)

Van Slyke estimation of amino-N

The estimation of free amino groups in proteins by the method of Van Slyke is complicated by the slow reaction of nitrous acid with other groups (e.g. the guanidyl group of arginine). For this reason the most satisfactory procedure

consists in plotting the N_2 evolved against the time of reaction and extrapolating the final portion of the curve, due to side reactions, to zero time [Kekwick & Cannan, 1936; Rutherford *et al.* 1937]. The curve shown (Fig. 1) was obtained with a micro-volumetric apparatus. If the molecular weight of the material is

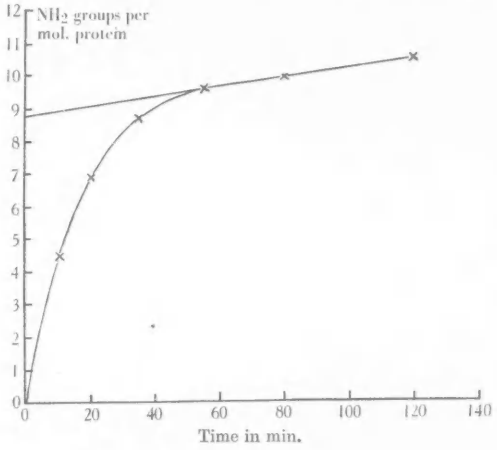

Fig. 1.

taken to be 18,000 it indicates the presence of nearly nine amino groups per molecule, and, on the assumption that only one of these is an *a*-amino group, of eight residues of lysine. The value of 5·8%, which was previously obtained for the lysine content of the substance, corresponds to seven residues per molecule.

Solubility of the material; its separation into two active fractions

It was stated by Roberts [1937] that lysozyme was moderately soluble in water but more soluble in dilute acetic acid, being partially precipitated from the latter solution on neutralization. A closer examination of the solubility showed that this was due to the presence of a small amount of an insoluble component. The material dissolved to the extent of about 90% in ten times its weight of water, but the residue was sparingly soluble, and after washing well with water dissolved, according to a micro-Kjeldahl estimation, only to the extent of 0·024% at room temperature. Its solubility was relatively small over a wide range of *p*H—4·5–10·5—but it was soluble, except for a negligible residue, in 5% NaCl solution. For this reason concentrated solutions of the original substance, which had been cleared by centrifuging, became slightly opalescent on dilution. The opalescence disappeared on the addition of a drop of NaCl solution.

A further small amount of protein separated from the soluble fraction, which comprised the main bulk of the material, on dialysing its solution, in fish swim bladders, against distilled water, at 0–5°, for 3 days. After centrifuging, the activity of the protein in the resulting clear solution was not distinguishable, by the present method of estimation, from that of the original material. It was reported to be homogeneous in the ultracentrifuge and to have a sedimentation

constant of $1{\cdot}8 \times 10^{-13}$. The protein could not be obtained from the dialysed solution by precipitation with acetone, or evaporation to dryness, without some denaturation. It was completely precipitated by ammonium sulphate between 45 and 66 % saturation. Fig. 2 shows the amount of protein in solution plotted

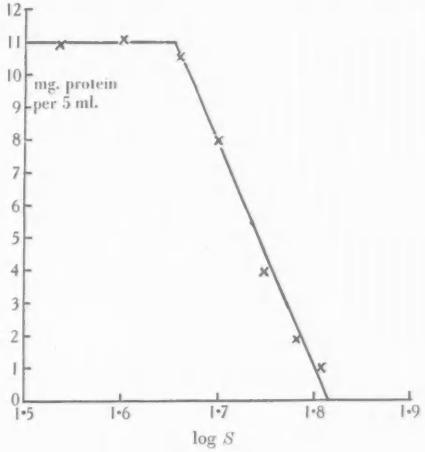

Fig. 2.

against the logarithm of the ammonium sulphate concentration (log S), at pH 4·6. Estimations were carried out by adding an equal volume of 5 % trichloroacetic acid to the solutions, heating to 70° for 5 min., washing the precipitate at the centrifuge until free of ammonia, and determining its total N by the micro Kjeldahl method.

The activity of the insoluble fraction appeared to be about one-third of that of the original substance. Ultracentrifugal measurements showed that it was less homogeneous and contained material of molecular weight lower than 18,000. Its sedimentation constant was given as $1{\cdot}63 \times 10^{-13}$. This, together with the fact that it gave a positive nitroprusside test for sulphydryl groups and that its nitrogen and sulphur contents (N 16·1, S 3·1 %) were very similar to those of the original material, suggested that it might consist of soluble, active, lysozyme adsorbed on "denatured" lysozyme formed during the preparation. No support, however, was obtained for this view from preliminary immunological experiments which were kindly carried out by Dr B. G. Maegraith in the School of Pathology. An antiserum was obtained from rabbits injected with a solution of lysozyme from which the insoluble fraction had been removed by dialysis and centrifuging. This showed a definite precipitin reaction with the antigen solution at a dilution of the latter of 10^4, but gave no reaction with the insoluble fraction at a dilution of 10^3. If the latter contained an amount of adsorbed soluble protein corresponding to its activity a positive reaction would have been expected under these conditions. This preliminary result thus indicates that the lytic activity is connected with two different proteins.

Electrometric titration

In order to obtain more information about the amphoteric nature of the preparation and the relation of the insoluble fraction to the rest of the material electrometric titrations were carried out.

Experimental

The apparatus consisted of a hydrogen electrode in a rocking cell, and was very similar to that used by Harington & Neuberger [1936] for the titration of insulin. It was standardized against 0·1 *N* HCl, the *p*H of this solution being taken as 1·096 [Scatchard, 1925].

Solutions of the soluble protein were prepared for titration in the following way: 600 mg. of lysozyme, prepared by the method of Roberts, were dissolved in 65 ml. of water, the insoluble fraction removed by centrifuging and the clear solution dialysed in a fish swim bladder against distilled water, at 0–5°, for 72 hr. The small amount of protein which separated during the dialysis was centrifuged off, and 12 ml. of the resulting solution, containing 70–80 mg. of protein, were used for each titration. A certain amount of the material (*c.* 15 %) appeared to be lost during dialysis. The protein concentrations of these solutions were determined by evaporating 5 ml. portions to dryness, drying at 110°, and weighing.

The insoluble fraction was washed well with water, redried with acetone and ether, and about 75 mg. portions, in 12 ml. of water, used for each titration.

Results

For several reasons, but especially because of the difficulty of making adequate blank corrections, accurate titrations are usually confined to a *p*H range of approximately 2·5–11·5.

In the case of egg albumin [Kekwick & Cannan, 1936] and insulin [Harington & Neuberger, 1936] about 80 % of the total acid binding took place at *p*H 2·5, but an end-point was obtained in the first case by extending the range to below *p*H 2·0 and in the second case by titrating in 80 % alcohol.

In the alkaline range the situation is less satisfactory. The guanidyl group of arginine cannot be completely included in the range to which accurate titration is limited, but, nevertheless, in the titration of insulin Harington & Neuberger found that, probably because no activity correction could be made, the base binding at *p*H 11·5 appeared unreasonably high in comparison with the analytical data.

In the present case the blank corrections were calculated on the assumption that the concentration of hydrogen ion was equal to its activity. Under the conditions of measurement a small amount of denaturation occurred and it was not possible to keep the solutions quite clear. This was probably a surface phenomenon: Roberts [1937] remarked on the ease of surface denaturation of lysozyme. Nevertheless, it appeared unlikely that any measurable change in the acid- and base-binding capacities of the material occurred, as the titrations in both the acid and alkaline ranges were reversible. After titration, especially in the alkaline range, the material appeared to be slightly less active than it was originally, but owing to the inaccuracy of the method of estimation it was only possible to say that no great loss in activity had occurred. Lowering the temperature of titration from 25 to 20° had very little effect on the denaturation.

Fig. 3 shows titration curves of dialysed solutions of the soluble protein at 25 and 20°, and an acid titration curve in 70 % alcohol at 25°. The figures for one titration are given in Table I. The acid- and base-binding per molecule have been calculated for a molecular weight of 18,000.

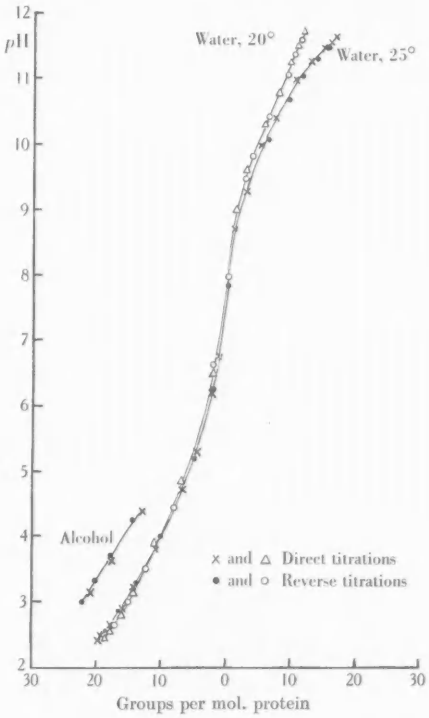

Fig. 3.

The *p*H of the dialysed solution should be close to the isoelectric point of the protein. This lay in a region where there was little buffering, and was 7·85 at 25° and 7·98 at 20°.

As would be expected the change in temperature made practically no difference to the results in the acid range. There was no indication of a maximum acid-binding at *p*H 2·5 in water, and at this point the curve shows the binding of about 19 groups per molecule of protein. It would be expected from the analytical data that there would be relatively little buffering in the range in which histidine is titrated, and in fact about 4 groups were titrated between the isoelectric point and *p*H 5·5.

Titration in 70 % alcohol was only possible in the acid range at *p*H values lower than 4·5, owing to the limited solubility of the protein in this solvent. There is difficulty concerning the definition of acidity in aqueous alcoholic solutions, and the term *p*H is used, in this case, in the sense indicated by Neuberger [1934]. The curve obtained lay about one *p*H unit above that given

Table I. *Titration of dialysed lysozyme solution*

A. With acid. 80 mg. protein (moisture and ash free); 12 ml. water. Temp. 25°.

ml. 0·1 *N* HCl	*p*H	ml. 0·1 *N* HCl corrected	Equiv. 10^5 H^+ bound per g. protein	Equiv. H^+ bound per g. mol. protein
—	7·850	—	—	—
0·05	6·765	0·050	6·2	1·1
0·10	6·195	0·100	12·5	2·2
0·20	5·327	0·199	24·9	4·5
0·30	4·718	0·298	37·2	6·7
0·50	3·821	0·481	60·1	10·8
0·70	3·211	0·622	77·7	14·0
0·90	2·848	0·717	89·6	16·1
1·10	2·636	0·797	99·6	17·9
1·30	2·484	0·864	108·0	19·4
1·40	2·416	0·886	110·8	19·9

Reversed titration

ml. 0·118 *N* NaOH	*p*H	ml. 0·1 *N* HCl corrected	Equiv. 10^5 H^+ bound per g. protein	Equiv. H^+ bound per g. mol. protein
0·20	2·585	0·810	101·3	18·2
0·40	2·822	0·720	90·0	16·2
0·60	3·237	0·611	76·4	13·7
0·80	4·024	0·443	55·4	10·0
1·00	5·208	0·219	27·4	4·9
1·10	6·190	0·102	12·7	2·3

B. With alkali. 80 mg. protein; 12 ml. water. Temp. 25°.

ml. 0·118 *N* NaOH	*p*H	ml. 0·1 *N* NaOH corrected	Equiv. 10^5 OH^- bound per g. protein	Equiv. OH^- bound per g. mol. protein
0·05	8·712	0·058	7·2	1·3
0·10	9·304	0·115	14·4	2·6
0·20	9·981	0·224	28·0	5·0
0·30	10·404	0·321	40·1	7·2
0·50	10·976	0·466	58·2	10·5
0·70	11·285	0·570	71·2	12·8
0·90	11·486	0·648	81·0	14·6
1·00	11·538	0·710	88·7	16·0
1·10	11·617	0·730	91·3	16·4

Reversed titration

ml. 0·1 *N* HCl	*p*H	ml. 0·1 *N* NaOH corrected	Equiv. 10^5 OH^- bound per g. protein	Equiv. OH^- bound per g. mol. protein
0·20	11·486	0·672	84·0	15·1
0·40	11·318	0·604	75·5	13·6
0·60	11·072	0·526	65·7	11·8
0·80	10·675	0·429	53·6	9·6
1·00	10·032	0·280	35·0	6·3

in aqueous solution, and at *p*H 3·0, where the acid-binding under these conditions would be expected to be nearly complete, the latter corresponded to 22–23 groups.

In the alkaline range no indication of maximum base-binding was obtained at *p*H 11·5. At this point the curve at 25° corresponded to binding by about 15 groups and that at 20° by about 11 groups per molecule of protein. This difference may be assigned to the temperature coefficients of the dissociation constants of the phenolic and guanidyl groups of tyrosine and arginine respectively.

The titration of the insoluble fraction from the original lysozyme was only possible below *p*H 4·5 and above *p*H 11. At *p*H 4·5 the acid-binding differed very little from that of the soluble protein, but with increasing acidity it showed greater acid-binding, the value at *p*H 2·5 being 24 groups per 18,000 g. At *p*H 11·5 the base-binding corresponded to about 21 groups. Thus at these *p*H values it appeared to bind about five more equivalents of acid and base than the soluble protein.

Discussion

The colorimetric estimations showed that lysozyme prepared by the method of Roberts contained a considerable quantity of arginine and lysine and an unusually large proportion of cystine. In spite of its apparent homogeneity in the ultracentrifuge, however, the lysozyme prepared by this method is not a single protein: a certain fraction of it has a much lower solubility, a lower activity and a somewhat larger acid- and base-binding capacity, than the rest. No material is present of molecular weight higher than 18,000, but while the main, soluble, portion is homogeneous as regards molecular size, some inhomogeneity is revealed in the insoluble fraction due to particles of lower molecular weight. The latter are not present in sufficient quantity in the original material to be detected in the ultracentrifuge.

These facts would be understandable if the active proteins in both fractions were very similar in molecular size and amphoteric properties, but the insoluble fraction also contained products of partial degradation. Immunological experiments by Roberts *et al.* [1938] indicated that egg, cat and human lysozymes were not identical; but the presence of two non-identical active proteins in material obtained from one source, which is suggested by the preliminary experiments carried out in this case, is more surprising. Further experiments are required in this connexion.

Summary

1. Lysozyme prepared by the method of Roberts appeared to be homogeneous in the ultracentrifuge and to have a molecular weight of about 18,000. A colorimetric estimation of certain amino-acids and a Van Slyke estimation of amino-nitrogen were carried out, and arginine was isolated in the form of its flavianate.

2. The solubility of the material subsequently showed that it was not completely homogeneous but that a fraction could be separated from it of lower activity and much lower solubility than the rest.

3. Electrometric titration of the soluble lysozyme showed that this was a basic protein with an acid-binding capacity not far from 23 groups per molecule. At *p*H 11·5 the base-binding in water, at 25°, corresponded to about 15 groups per molecular. The acid- and base-binding capacities of the insoluble fraction were somewhat larger.

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